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PRINCIPAL INVESTIGATOR: Joy L. Ware, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, Virginia 23298-0568

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**6. AUTHOR(S)**
Joy L. Ware, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Virginia Commonwealth University
Richmond, Virginia 23298-0568

E-Mail: jlware@hsc.vcu.edu

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**13. ABSTRACT** *(Maximum 200 Words)*

The overall hypothesis to be tested was that qualitative and/or quantitative differences in the expression of the Wilms Tumor Gene (WT1) would alter the behavior of human prostate cancer cells, especially with regard to proliferation. During this study we discovered a novel transcript of WT1 (TR-WT1) consisting of portions of intron 5 and exons 6-10. Analysis of prostate cancer cell lines transfected with wild-type WT1 (+17,+KTS) revealed that induction of WT1 expression could suppress transcription of the insulin-like growth factor receptor (IGFR) promoter, which was linked to decreased IGF responsiveness. In contrast, transfection of the TR-WT1 did not affect endogeneous transcription of either IGFR or the epidermal growth factor receptor (EGFR). M2205 cells transfected with TR-WT1 (-KTS) doubled significantly faster in vitro and were more tumorigenic in vivo than controls or cells transfected with TR-Wt1 (+KTS). Analysis of microdissected human prostate cancers revealed that WT1 or TR-WT1 mRNA was detected in 18/32 malignant glands. Collectively, these results suggest that WT1 and/or TR-WT1 products may play an unusual role in prostate cancer behavior.

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## Table of Contents

(4) Introduction

- The overall hypothesis tested in this research project is that quantitative and/or qualitative differences in the expression of the Wilms' Tumor 1 (WT1) gene alter the balance between proliferation and apoptosis in human prostate cancer cells. As a consequence of such changes, the aggressiveness of prostatic cancer may be altered. Our objective was to alter expression of specific isoforms of wild-type WT1 or a novel truncated form of WT1, TR-WTI, in human prostate cancer cells by stable transfection with the appropriate constructs, and evaluate the biological impact. In fact, we discovered this novel WT1 transcript TR-WT1, during the first year of this DOD funded project. We developed quantitative real time PCR assays for both WT-1 and TR-WT1, permitting accurate measurement of WT1 expression in these cells. The behavior of these transfected cells, and appropriate controls, was evaluated in vitro and in vivo in athymic nude mice. In parallel, we initiated study of snap frozen microdissected human residual prostate tissue samples (paired tumor and benign glands) from patients undergoing radical prostatectomy. Analysis by RT-PCR confirmed that both wild type and TR-WT1 are expressed in some malignant prostate glands.

(5) Body of Report

- This final report describes our accomplishments over the entire interval of the award in relation to the specific tasks described in our statement of work.

TASK 1: Create and characterize stable transfectants of the target cells that (A) overexpress WT1 RNA and protein or (B) have reduced WTI expression.

As we reported previously, transfection of the P69SV04)TAg cells with the full length +17, +KTS isoform of WT1 generated cells expressing the expected product. Efforts to obtain stable transfectants with the +17, -KTS isoform failed, despite repeated attempts.

However, as detailed in our publication included in the appendix (Damon et al, 2001), overexpression of +17, +KTS WT1 constructs significantly inhibited transcription from IGF-1 receptor promoters linked to luciferase reporter constructs, (see Figure 1A reproduced from Damon et al, 2001.) In addition, endogenous transcription of the IGFR gene was reduced. (Fig. 1B) Thus overexpression of wild type WT1 can profoundly reduce IGF-IR expression, a receptor implicated in control of prostatic cancer cell proliferation and progression.

**Figure 1A**

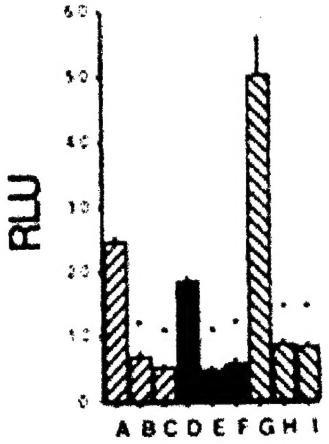



Fig. 1A. IGF-IR promoter activity in P69 cells and two clonal lines derived by stable transfection of P69 with a WT1 (+KTS) expression vector and designated B7 and D16. A–C, Full-length promoter; D–F, 5'-flanking regions; G–H, 5'-UTR. A, D, and G, P69 cells; B, E, and H, B7 cells; C, F, and I, D16 cells. The relative light units (RLU) of luciferase activity per mg protein was controlled for transfection efficiency as described in *Materials and Methods.* *,$P$ <0.0001 for B and C *vs.* A, for E and F *vs.* D, and for H and I *vs.* G.

**Figure 1B**

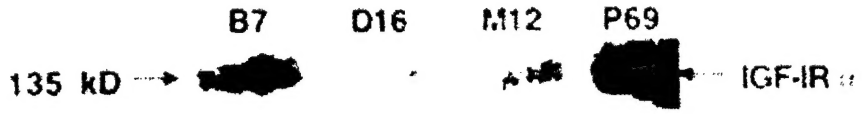
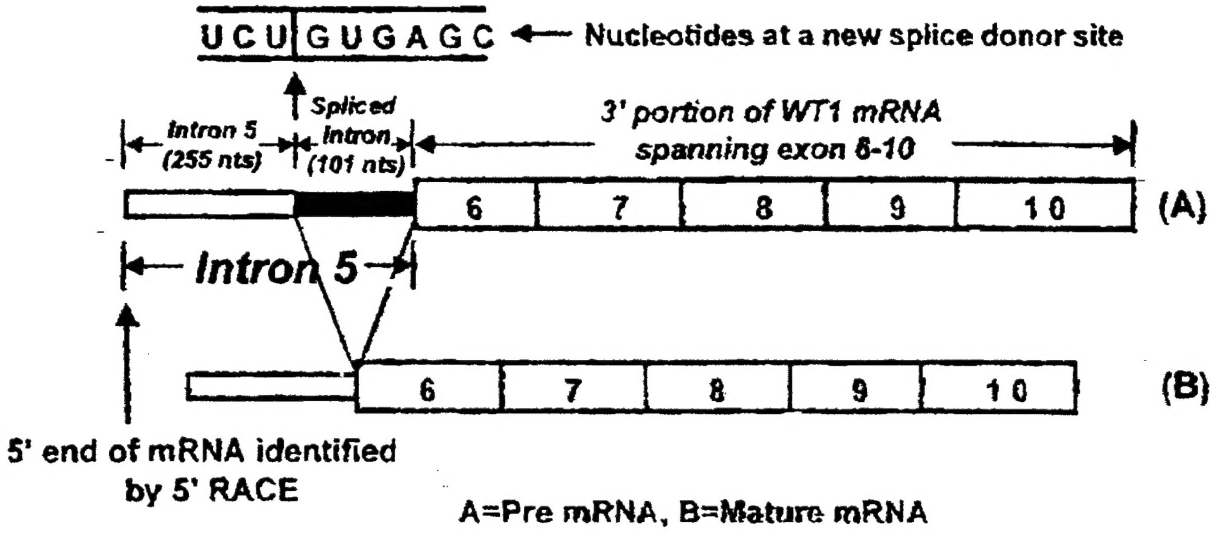



Fig. 1B. Western immunoblot of M12, P69, D16, and B7 lysates probed with antibody to the α-subunit of the IGF-IR. Each lane contains 100 $\rho$g protein. Note the marked decreased in IGF-IR expression in the metastatic M12 line and the B7 and D16 clones, derived by transfection of the P69 line with a WT1 expression vector, compared with P69 parental line. (From Damon et al, 2001, see appendix)

During the first year of this project we discovered and characterized a novel transcript of WT1, consisting of portions of intron 5 and exons 6-10 of WT1 illustrated in Figure 2, reproduced from (Dechshukhum et al, 2000). We created expression vectors from the pcDNA 3.1 vector containing the DNA encoding this novel short transcript (2.1 kb) and successfully transfected P69SV40TAg cells, the tumorigenic but non-metastatic M2205 cells, and the PC-3 line with their constructs. Each line was transfected with the +KTS or the –KTS isoform of the short transcript. Increased expression of the construct was confirmed in each of the transfected lines by quantitative RT-PCR (Figure 3) and northern blot analysis.

**Figure 2**




Schematic diagram of 2.1 kb of truncated WT1 mRNA in human prostate cell lines. The transcript appears to consist of 225 bp of intron 5 plus exons 6 through 10 of WT1. A 101-bp portion of intron 5 has been spliced out to make a mature mRNA. The resultant WT1 transcript is approximately 2,000 bp, similar to the size of the similar truncated transcript detected by northern blot. Note that the new splice donor site shows partially preserved consensus sequence. (From Dechshukhum et al, 2000)

A quantitative RT-PCR assay for both wild-type WT1 and TR-WT1 was developed (Dumar et all, 2000, abstract; manuscript in progress). This quantitative real-time PCR assay is based on two WT1-specific in vitro-generated RNA as calibrators. Serial dilution of these calibrators revealed a 5 log linear dynamic range ($5x10^1$ to $5x10^6$ copies/reaction, $R^2$=0.9964 for WT1 and $R^2$=0.9975 for TR-WT1). Dilution of the calibrators with total RNA from non-WT1-expressing cells showed decreased sensitivity without affecting the linear range. Thus we developed and validated a sensitive, reliable method for quantitation of both of these WT1 transcripts suitable for prostate tissues, as well as cell lines.

We applied this quantitative assay to the TR-WT1 transfectants of P69SV40TAg, M2205, and PC-3 that we created. Figure 3 demonstrates the TR-WT1 copy number/mg of total RNA in each of these lines.

**Figure 3**

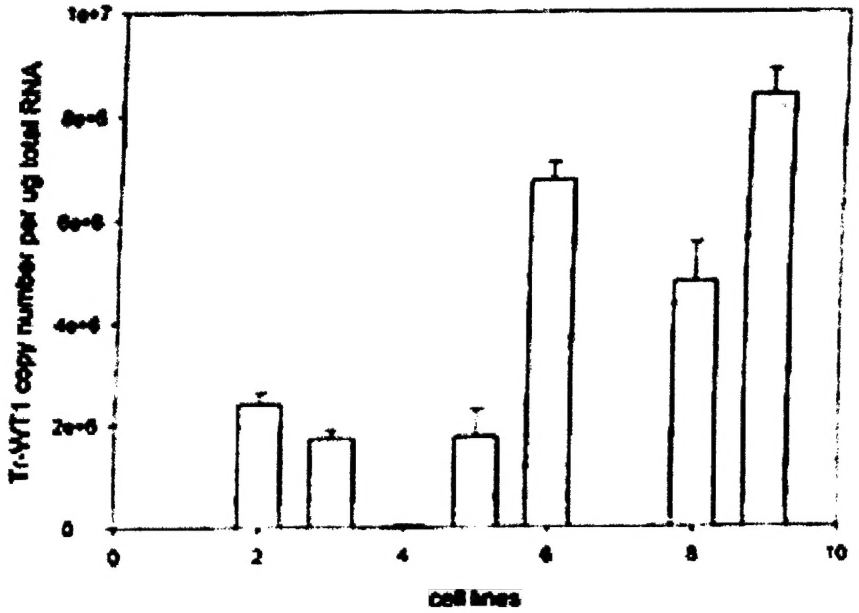
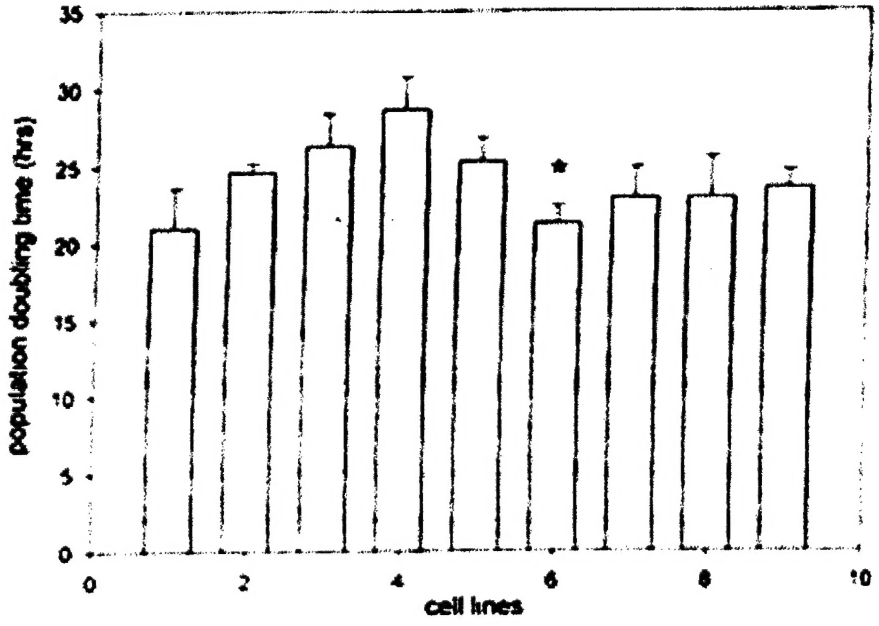



Figure 3: Quantitative RT-PCR analysis of TR-WT1 expression in controls and cell lines transfected with either TR-WT1 (+KTS) or TR-WT1 (-KTS). Horizontal axis: identity of cell lines; Cell line No.1 P69 Control; Cell line No. 2 P69 +KTS; Cell line No. 3 P69 –KTS; Cell line No. 4 M2205 Control; Cell line No. 5 M2205 +KTS; Cell line No. 6 M2205 –KTS; Cell line No. 7 PC3 Control; Cell line No. 8 PC3 +KTS; Cell line No. 9 PC3 –KTS.

**Figure 4**

Population Doubling Time in Vitro of Control and TR-WT1 Transfected Cells



Population Doubling Time

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mean | 21 | 25 | 26 | 29 | 25 | 21 | 23 | 23 | 24 | Hours |
| SD | 2.6 | 0.6 | 2.1 | 2.1 | 1.5 | 1.2 | 2.0 | 2.6 | 1.2 | |

Cells were plated in 60mm dishes in triplicate. Cells were collected with trypsin and counted in a hemacytometer each day over a six day interval. Cell line No.1 P69 Control; Cell line No. 2 P69 +KTS; Cell line No. 3 P69 –KTS; Cell line No. 4 M2205 Control; Cell line No. 5 M2205 +KTS; Cell line No. 6 M2205 –KTS; Cell line No. 7 PC3 Control; Cell line No. 8 PC3 +KTS; Cell line No. 9 PC3 –KTS.
*Significantly different from control, $p<0.05$, unpaired student and test.

TASK 2: Evaluate the transfectants created during Task 1, in vivo in male athymic nude mice.

Orthotopic injection of P69SV40TAg cells (which are immortal but nontumorgenic) did not produce tumors, as expected. However, the orthotopic injection of P69SV40TAg cells transfected with +17, +KTS WT1 also failed to produce tumors. This is consistent with our previously reported finding after subcutaneous injection.

In contrast, transfection of the tumorgenic, nonmetastatic M2205 line with plasmids encoding the truncated WT1 transcript (TR-WT1) did affect tumorigenicity in vivo. All mice injected intra-prostatic with the control M2205 cells formed tumors within the 3 month observation interval. In contrast 0/3 mice injected with TR-WT1 (+KTS) failed to form tumors. However 3/3 mice injected with TR-WT1 (-KTS) formed large intraprostatic tumors prior to the end of the observation period. In fact, these mice were sacrificed early, due to catexic and distress from the tumors.

Because it is impossible to measure intraprostatic tumor growth in vivo, we also assessed the behavior of these TR-WT1 overexpressing M2205 cells in athymic nude mice injected subcutaneously. As the following table indicates, mice injected with M2205 cells expressing TR-WT1 (-KTS) were highly tumorigenic. The observed growth in vivo is consistent with the decreased population doubling time of M2205 (-KTS) in vitro (Figure 4).

Tumorigenicity of M2205 cells and TR-WT1 overexpressing M2205 after subcutaneous injection into athymic nude mice.

**Table 1**

| Cells | Incidence | Latent Interval |
|---|---|---|
| Control M2205 | 1/5 | 24 days |
| TR-WT1 (+KTS) | 0/5 | ——— |
| TR-WT1 (-KTS) | 4/5 | 18 days |

(Previous experience has shown that M2205 cells do not grow well after subcutaneous injection. Collectively, these data indicate that increased expression of TR-WT1 (-KTS) increases the tumorigenicity and apparent growth rate of the M2205 cells at 2 different sites in vivo. Thus the behavior of the TR-WT1 (-KTS) cells are different from parental M2205 cells in vivo.)

Using both RT-PCR and northern blot analysis, we examined expression of EGFR and IGFR message in each of the three sets of P69SV40TAg, M2005, and PC-3 cells created by transfection of the cDNA encoding TR-WT1 (+KTS), TR-WT1 (-KTS) or empty plasmid (control). These experiments were each repeated three times, with essentially the same result: increased expression of TR-WT1 (+KTS) or TR-WT1 (-KTS) did not affect the expression of the endrogenous EGFR and IGFR genes.

TASK 3: Quantitate WT1 mRNA expression in human primary prostate cancers of different histopathologic grade and stage. Evaluate WT1 protein expression by immunohistochemistry.

Using the Arcturus Laser Capture Microdissection Apparatus, we began our recovery of paired benign and malignant prostate glands from each case. Detailed methodology is provided in the copy of the manuscript indicated in the appendix. Table 2, from that manuscript, summarizes our findings.

In brief, both the wild type and the truncated WT1 (TR-WT1) transcripts were detected by RT-PCR among malignant prostate glands. Either the wild type WT1 or the truncated WT1 was expressed in 18/32 malignant glands. Glands expressing wild type WT1 continued to express EGFR and IGFR MRNA.

However, some morphologically benign glands also expressed WT1. At present, it is not clear whether this represents a "field effect", expression in benign glands in transition to malignancy, or simply expression in benign glands.

Unfortunately, due to circumstances having nothing to do this DOD project, all projects involving human subjects at VCU were closed by NIH at the end of December, 2000, pending administrative review and re-organization. This was due to administrative deficiencies in the VCU IRB program.

As a consequence, we were required to stop all of our work on Aim 3 of this project, until this project, along with all others at VCU, could be re-reviewed by an outside agency.

Although we submitted the request for re-review as soon as possible (early February 2000), as of January 2001 the re-review had not occurred. During this time no DOD funds were expended in support of Aim 3, and no further work was allowed. As a result of our compliance with this directive, a substantial amount of money could not be spent as planned and will be returned to the DOD at the termination of this project.

Table 2: Summary of RT-PCR result of wild type and truncated WT1 transcripts expression in human primary prostate cancer.

| Clinico-Pathology Data | | | | | RT-PCR of WT1 | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Age | Race | Gleason Score | PSA Level | Wild Type | | Truncated | |
| | | | | | B | T | B | T |
| 28 | 60 | CA | 6 | 5.2 | - | - | - | + |
| 35 | 61 | CA | 6 | 1.2 | - | - | - | + |
| 40 | 70 | AA | 5 | 1.6 | ND | ND | - | - |
| 45 | 66 | CA | 7 | 4.73 | ND | ND | - | + |
| 60 | 60 | CA | 6 | 4.16 | + | - | - | - |
| 62 | 58 | CA | 6 | 4.1 | - | + | - | - |
| 19 | 62 | CA | 5 | 4.44 | - | + | - | - |
| 22 | 70 | CA | 8 | 12.5 | - | - | - | - |
| 96 | 57 | AA | 7 | 4.9 | - | - | - | - |
| 24 | 59 | CA | 6 | 7.7 | - | + | - | - |
| 112 | 66 | AA | 7 | 10 | + | + | - | - |
| 123 | 56 | AA | 7 | 19 | - | + | - | - |
| 57 | 66 | CA | 5 | 4.4 | + | + | - | - |
| 63 | 59 | CA | 5 | 4.8 | - | - | - | - |
| 70 | 63 | CA | 6 | 5.8 | + | + | - | - |
| 76 | 63 | CA | 7 | 2.4 | - | - | - | - |
| 130 | 58 | AA | 7 | 12.6 | - | - | - | - |
| 138 | 60 | AA | 5 | 6.2 | - | - | - | - |
| 68 | 69 | CA | 7 | 5.8 | - | - | - | - |
| 85 | 62 | CA | 6 | 10.1 | - | + | - | - |
| 86 | 68 | CA | 5 | 7 | - | + | - | - |
| 89 | 66 | CA | 4 | 4.47 | + | - | - | - |
| 91 | 70 | CA | 8 | 7 | + | - | - | - |
| 141 | 59 | AA | 7 | 5.1 | - | - | - | - |
| 154 | 62 | AA | 9 | 7.09 | - | - | - | - |
| 92 | 66 | CA | 6 | 9.8 | - | + | - | - |
| 94 | 69 | CA | 6 | 9.3 | - | + | + | - |
| 95 | 63 | CA | 8 | 7.34 | - | + | - | + |
| 98 | 60 | CA | 6 | 4.2 | + | + | - | - |
| 99 | 62 | CA | 6 | 1.2 | + | + | + | + |
| 100 | 71 | CA | 6 | 13.27 | - | + | - | + |
| 75 | 51 | CA | 6 | 14.3 | + | - | - | - |

*B = benign glandular epithelium, T = malignant glandular epithelium, ND = study was not successful due to diminished integrity of RNA, CA = Caucasian American, AA = African American

(6) Key Research Accomplishments

- Construction of human prostate cancer cell lines transfected with the +17, +KTS and the –17, +KTS isoforms of wild-type WTI.
- Detection and characterization of novel transcript (approximately 2.1 kb) of the WT gene. (Dechshukhum et al, Mol. Diagnosis 5:117-128, 2000).
- Cloning and sequencing of this novel WTI transcript from the M12 prostate cancer cell line. This transcript includes a portion of intron 5 at the 5 end and extends from exon 6 through exon 10. (Dechshukum et al, Mol. Diagnosis 5:117-128, 2000).
- Analysis by RT-PCR of laser capture microdissected benign and malignant human prostate cancers revealed that both the wild-type and the novel with transcript were expressed in human prostate cancers. Either wild type or truncated WTI transcripts were found in malignant glands in 18/32 cancer specimens. (Manuscript submitted)
- However, expression of wild-type WT1 was seen in 10/32 benign glands, located in close proximity to the malignant glands. Whether this indicates a "field effect" or that expression of WTI in prostate may increase with age is not known.
- Construction of three different human prostate cancer cell lines overexpressing the +KTS or –KTS isoform of the truncated WTI: P69SV40T, M2205 (a tumorigenic subline of P69SV40T), and PC-3 cells were each transfected in parallel with cDNA encoding the above isoforms of the novel transcript.
- Development of quantitative real-time PCR assays for (1) wild-type WTI and (2) truncated WT-I (Tr-WTI). (Dumar et al, abstract, Association of Molecular Pathology).
- Overexpression of the KTS(-) isoform of truncated WTI significantly decreases the population doubling time M2205 in vitro and appears to increase tumorigenicity (and possibly in vivo growth rate). Mice injected orthotopically (intra-prostatic) with M2205 cells over-expressing KTS(-) Tr-WTI develop larger tumors, earlier, than control mice.
- Thus far, no mice injected orthotopically or subcutaneously with the M2205 KTS(+) Tr-WTI overexpressing cells has developed tumors.
- Prostate cancer cells expressing higher levels of Tr-WT1, i.e., M12 cells have reduced levels of endogenous IGFR and significantly less capacity to activate the IGFR promoter in transient transfection assays. (Damon et al, Endocrinology 142:21-27, 2001).
- Overexpression of wild-type (+17, +KTS) WTI in P69SV40TAg cells significantly reduced transcription of the IGF receptor gene, both endogenous expression and in transient transfection assays. (Damon et al, Endocrinology 142:21-27, 2001).

(7) Reportable Outcomes:

<u>Published Manuscripts</u>


- Ware, JL: Growth factor network disruption in prostate cancer progression. Cancer Metastasis Reviews. 17:443-447, 1999.
- Dechsukhum, C, Ware, JL, Ferreria-Gonzalez, A, Wilkinson, DS, and Garrett, CT: Detection of a novel trumcated WTI transcript in human neoplasia. Mol. Diagnosis. 5:117-128, 2000.

- Damon, SE, Plymate, SR, Carroll, JM, Sprenger, CC, Dechsukhum, C, Ware, JL, and Roberts, CT, Jr: Transcriptional regulation of IGF-1 receptor gene expression in prostate cancer cells. Endocrinology, 142:21-27, 2001.

Published Abstracts

- Dechsukhum, C, Ware, JL, Ferreira-Gonzalez, A, Wilkinson, DS, and Garrett, CT. Detection of novel WT1 transcripts with an intronic start site in human prostate cancer cell lines. Proc. Natl. Assoc. for Cancer Res. 40:367, 1999. (Previously provided)
- Damon, SE, Carroll, J, Roberts, CT, Jr., Dechsukhum, C, Ware, JL, Springer, CC, and Plymate, SR: Suppression of type 1 insulin-like growth factor receptor (IGF-IR) in a malignant prostate cell line involves sequences upstream of the IGF-IR gene transcription start site. Abstract P1-609, presented at the Endocrine Society Annual Meeting, 1999. (Previously provided)
- Dumur, CI, Dechsukhum, C, Garrett, CT, Wilkinson, DS, Ware, JL, and Ferreria, A: A real-time RT-PCR assay for qualification of different WTI transcripts in cancer. Assoc. for Mol. Pathology, 2000.

Manuscripts Submitted

- Dechsukhum, C, Garrett, CT, Ferreria-Gonzalez, A, Supanatsetakal, N, Burks, RT, Morgan, W, Wilkinson, DS, and Ware, JL: Expression of WTI transcripts in prostate cancer. Submitted.

Patent Applications and Approval

- "A nucleic acid marker for cancer." Inventors: Joy L. Ware, Chavaboon Dechsukhum, and Carl T. Garrett. Provisional application no. 60/118, 749, filed Feb. 4, 1999.
- "A nucleic acid marker for cancer." Inventors: Joy L. Ware, Chavaboon Dechsukhum, and Carl T. Garrett. Patent Application Serial Number 09/434, 620, issued Note of allowance and approval of claims.

Degree Obtained

- Chavaboon Dechsukhum, MD, earned his PhD degree in 2000 based upon work funded by this proposal.

(8) Conclusions:

Overall, the significance of this work lies in three areas. First, a novel WT1 transcript was identified in both prostate cancer cell lines and some malignant prostate glands.

Second, the expression pattern of both the wild type and the truncated WT1 transcript has the potential to become an additional prognostic marker for prostate cancer. Whatever the significance of WT1 expression for prostate cancer biology, it is clearly different from the traditional concept of WT1 as a tumor suppressor.

Third, both wild type WT1 and the truncated WT1 product can affect the behavior of some prostate cancer cell lines. However, whether an effect is seen is dependent on the isoform of WT1, the cell line, and the cellular context.

(9) References:


- Dechsukhum C, Ware JL, Ferreira-Gonzalez A, Wilkinson DS, and Garrett CT: Detection of a novel truncated WT1 transcript in human neoplasia. Molecular Diagnosis. 5, 2000.
- Damon SE, Plymate SR, Carroll JM, Sprenger CC, Dechsukhum C, Ware JL, and Roberts, Jr. CT: Transcriptional regulation of insulin-like growth factor-I receptor gene expression in prostate cancer cells. Endocrinology. 142, 2000.
- Dechsukhum C, Garrett CT, Ferreira-Gonzalez A, Supanatsetkul N, Burks RT, Morgan W, Wilkinson DS, and Ware JL: Expression of WT1 transcripts in human prostate cancers. Submitted.
- Dumur CI, Dechsukhum C, Garrett CT, Wilkinson DS, Ware JL, and Ferreira-Gonzalez A: A real-time RT-PCR assay for quantitation of different WT1 transcripts in cancer. Association for Molecular Pathology. Abstract, 2000.


Personnel Receiving Salary from this Award:

Joy L. Ware, PhD
Carleton T. Garrett, MD, PhD
Angela Moulton, BS
R. Tucker Burks, MD

# Detection of a Novel Truncated WT1 Transcript in Human Neoplasia

CHAVABOON DECHSUKHUM, MD, JOY L. WARE, PhD,
ANDREA FERREIRA-GONZALEZ, PhD, DAVID S. WILKINSON, MD, PhD,
CARLETON T. GARRETT, MD, PhD

*Richmond, Virginia*

**Background:** The Wilms' tumor 1 (*WT1*) gene encodes a transcription factor critical in urogenital development. Using a new model of prostate cancer progression that permits comparison of the cellular and molecular properties of increasingly aggressive sublines of simian virus 40 large T-antigen–immortalized human prostate epithelial cells within the same lineage, the role of *WT1* in tumorigenesis was investigated.

**Method and Results:** Using RT-PCR and northern blotting, we identified a novel truncated WT1 transcript in these prostate cancer cell lines. This 2.1-kb transcript consisted of the coding region of the zinc-finger domain of *WT1*, together with a portion of intron 5 at the 5′ end of the transcript. Furthermore, two peptides were detected by western blotting using antibodies to epitopes of the COOH terminus of *WT1*. Using RT-PCR, the 2.1-kb transcript was also detected in leukemia cell line K562, breast cancer cell line MCF7, and blood samples from patients with acute leukemia.

**Conclusion:** These novel findings in both cell lines and patient-derived specimens suggest this new *WT1* gene alteration has a potential role in the development of new diagnostic assays for some human malignancies.

**Key words:** WT1, prostate cancer, novel transcript.

The Wilms' tumor 1 (*WT1*) gene encodes a transcription factor [1,2] that is critical for normal urogenital development [3,4]. However, the role of *WT1* in prostate disease is not known. Decreased expression of *WT1* and increased expression of insulin-like growth factor type I (IGF-I) receptors have been reported in stromal cells from benign hyperplastic prostate tissue compared with stromal cells from normal or malignant prostate [5]. *WT1* regulates transcription of several growth factors and growth factor receptors implicated in prostatic growth control, specifically the epidermal growth factor receptor (EGFR) [6,7]; IGF-II [8,9], the IGF-I receptor (IGFR-I) [10,11]; and the androgen

*From the Department of Pathology, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, VA.*

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Reprint requests: Joy L. Ware, PhD, Department of Pathology, PO Box 980-662, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, VA 23298-0662. Email: JLWARE@hsc.vcu.edu



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receptor [12]. Given this relationship, the possible involvement of *WT1* in prostate cancer cell biological processes was investigated.

We previously established and characterized a new model of human prostate cancer progression that permits comparison of the cellular and molecular properties of increasingly aggressive sublines of simian virus 40 (SV40) large T-antigen–immortalized human prostate epithelial cells within the same lineage [13–15]. These cell lines show the following phenotypes, assessed by orthotopic and/or subcutaneous injection into athymic nude mice: immortal, nontumorigenic, tumorigenic but nonmetastatic, and consistently tumorigenic and metastatic, respectively. After determining that expression of EGFR [13] and IGFR [16] decreased dramatically in the metastatic M12 cells compared with the parental line, we investigated (1) whether *WT1* was expressed in any of these sublines, and (2) whether expression correlated with the previously reported alteration in the *in vivo* phenotype and/or the reduction in EGFR and IGFR. These investigations led to an unexpected finding with implications for the role of *WT1* in oncogenesis.

In this report, we show the existence of a novel 2.1-kb WT1 transcript in the previously mentioned model of human prostate cancer. Using RT-PCR, northern blot analysis, and 5′ rapid amplification of complementary DNA (cDNA) end (5′ RACE), we showed that these WT1 transcripts lack exons 1 through 5 and contain at their 5′ end sequences normally confined to intron 5 of the *WT1* gene, along with exons 6 through 10 at their 3′ end. The overall length was approximately 2.1 kb. The existence of this shortened WT1 transcript in both human tumor cell lines and leukemias suggests a new mechanism of *WT1*-mediated oncogenesis.

## Materials and Methods

### Human Tumor Cell Lines

Prostate cell lines were maintained in serum-free RPMI 1640 (CellGrow, Inc.) supplemented with dexamethasone (0.1 μM); insulin, transferrin, and selenious acid ([ITS] insulin, 5 μg/mL; transferrin, 5 μg/mL; and selenium, 5 ng/mL); epidermal growth factor, 10 ng/mL (Collaborative Research, Bedford, MA); and gentamicin, 0.05 μg/mL (Gibco/BRL, Rockville, MD). The parental P69SV40TAg cell line and the derivation of its tumorigenic (M2182, M2205) and metastatic (M12) sublines were previously described in detail [13–15].

Briefly, adult human non-neoplastic prostate epithelial cells were isolated from the prostate of a 63-year-old black man undergoing transurethral resection, and cells were immortalized by transfection with the SV40 T-antigen gene [13]. Rare tumors arising in two of 18 athymic nude mice were recovered and subjected to sequential *in vitro* and *in vivo* growth cycles to permit isolation of increasingly aggressive sublines [14,15].

The PC-3 human prostate carcinoma cell line was originally isolated from a prostate cancer bone metastasis [17]. PC-3 cells used in this study were obtained by J.L.W. from a PC-3 stock at Duke University (Durham, NC) and maintained in 10% fetal bovine serum (HyClone Laboratory Inc, Logan, UT). The K562 and MCF7 cell lines were purchased from American Type Cell Culture (Rockville, MD). The K562 cells were cultured in RPMI 1640 supplemented with 15% fetal bovine serum. MCF7 cells were grown in minimum essential medium eagle supplemented with ITS (insulin, 5 μg/mL; transferrin, 5 μg/mL; and selenium, 5 ng/mL); 10% fetal bovine serum; and gentamicin (0.05 μg/mL).

All cells were free of *Mycoplasma* organisms, as assessed by the Gen-Probe *Mycoplasma* T.C. Rapid Detection system (Gen-Probe, San Diego, CA).

### RNA Extraction

Total RNA was extracted from freshly isolated cultured cells and from frozen pellets of peripheral-blood leukocytes from two patients with acute leukemia (one patient, acute myelocytic; one patient, acute lymphocytic) using a phenol/isothiocyanate procedure, Ultraspec reagent and extraction protocol (Biotex Laboratory, Inc, Houston, TX) [18]. The samples of primary leukemia cells were obtained in accordance with an institutional review board–approved protocol for acquiring and banking residual clinical samples. Total RNA was dissolved in diethyl pyrocarbonate–treated double-distilled water (DEPC-treated water) and ribonuclease inhibitor RNasin, 1 U/μL (Promega Corp., Madison, WI), and stored at −80°C until use. Messenger RNA (mRNA) was prepared from total RNA by oligo (dT) column purification (Five Prime → Three Prime, Inc, Boulder, CO). One milligram RNA was applied to the column, and the mRNA fraction was

eluted with elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) according to the manufacturer's protocol [19]. The resulting mRNA was stored in DEPC-treated water in the presence of RNasin at −80°C until use.

## RT-PCR

Total RNA (0.7 μg) was used in an RT-PCR. The RT step was performed at 42°C in 75 mM KCl, 50 mM Tris (pH 8.3), 3.0 mM $MgCl_2$ (Gibco/BRL), 833 μM dNTPs (Promega Corp.), 2 μM primers, and 4.2 U/μL Moloney Murine Leukemia Virus (M-MLV) RT (Gibco/BRL) for 1 hour, followed by M-MLV heat inactivation at 95°C for 5 minutes. The cDNA was then transferred to a PCR master mix with a resulting final concentration of 56 mM KCl, 19.6 mM Tris (pH 8.3), 1.6 mM $MgCl_2$ (Perkin Elmer, Foster City, CA), 200 μM dNTPs (Promega Corp.), and 1 μM primers. Forty cycles of PCR were performed using 95°C denaturation for 1 minute, 60°C annealing for 30 seconds, and 72°C extension for 1 minute using GeneAmp PCR system 9600 Thermal Cycler (Perkin Elmer). The primer and probe sequences are listed in Table 1. PCR products were analyzed by electrophoresis through 1.5% agarose gel at 100 V for 3 hours, then transferred to Zeta Probe membrane (Bio-Rad Laboratory, Hercules, CA), cross-linked by UV light using Stratagene UV cross-linker (Stratagene, La Jolla, CA). Filters were hybridized with oligonucleotide probes end labeled with $^{32}P$ γ-dATP, 3,000 Ci/mmol (NEN Life Science Products, Inc, Boston, MA) at 50°C to 60°C for 6 to 12 hours. Autoradiographs were performed for 24 to 72 hours.

## Northern Blot Analysis

Northern blotting was used to determine the size of mRNA. Four micrograms mRNA were loaded onto an 0.8% formaldehyde agarose gel and electrophoresed at 120 V for 4 hours in formaldehyde gel running buffer, 0.02 M 3-[N-morpholino] propane sulfonic acid (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0) (Sigma, St Louis, MO). After electrophoresis, the gels were rinsed in DEPC-treated water for 10 minutes, followed by washing in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 1 hour (three times for 20 minutes). The samples were transferred overnight to nylon membrane, crossed-linked by UV light, and subjected to hybridization for 12 to 20 hours (73°C to 80°C annealing temperature). Filters were hybridized with RNA probes constructed from the 5′ and 3′ ends of *WT1*, as described next. Washing was performed using low-stringency buffer consisting of 1 × SSC, 0.1% sodium dodecyl sulfate (SDS), and high-stringency buffer consisting of 0.1 × SSC, 0.1% SDS, at 73°C to 80°C. Autoradiography was performed for 3 to 5 days.

The $^{32}P$ α-rUTP (3,000 Ci/mmol)–labeled RNA probes were generated by *in vitro* transcription using MAXI script T7/T3 or Strip-EZ kit (Ambion Inc, Woodward, TX). The templates were linearized plasmid using pCR-Script Amp SK (+) (Stratagene) containing cloned double-stranded RT-PCR fragment spanning nucleotides 579 (exon 1) to 1,159 (exon 5) for the 5′ probe or RT-PCR fragment spanning nucleotides 1,388 (exon 7) to 1,801 (exon 10) for the 3′ probe. The template sequences were confirmed as wild type by automated DNA sequencing on an ABI 373 DNA sequencer (Perkin Elmer) (data not shown).

## 5′ RACE Procedure

To map the 5′ region of the truncated WT1 transcript, we used the 5′ RACE procedure supplied by Gibco/BRL. Briefly, 0.5 μg total RNA or 0.05 to 0.1 μg mRNA were used to synthesize cDNA using an RT antisense primer located in exon 8 (5′-ACC TTC GTT CAC AGT CCT TG-3′). The cDNA was then subjected to a tailing reaction by terminal deoxynucleotidyl transferase for 10 minutes in the presence of 200 μM dCTP. The dCTP-tailed cDNA was then amplified in the first round of a heminested PCR procedure using an inner antisense primer located in exon 7 (5′-CTG CTG TGC ATC TGT AAG TG-3′) and the 5′ RACE Abridged Anchor Primer. PCR reactions were performed for 35 cycles using 95°C denaturation for 1 minute, 55°C annealing for 1 minute, and 72°C extension for 2 minutes in the presence of 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 μM dNTPs, and 400 nM primers.

The PCR product was then purified using a Centricon-100 column (Millipore Corp, Inc, Bedford, MA), transferring the whole PCR reaction (50 μL) to the spin columns to which 2 mL high-performance liquid chromatography (HPLC)-purified water (Fisher Scientific Co, Pittsburgh,

PA) had previously been added. Centrifugation was performed at 1,000*g* for 35 minutes. The second round of nested PCR was performed on diluted purified PCR product (100-fold in HPLC water) using a 3′ WT1-specific antisense primer located in exon 6 (5′-CGT TGT GTG GTT ATC GCT CT-3′) and a 5′ Abridged Universal Amplification Primer under the same conditions. The PCR product was then visualized under UV light on an ethidium bromide–stained 1.5% agarose gel. Southern blot was performed, and the filter was hybridized with a probe in exon 6 (5′-CCA CAG CAC AGG GTA CGA-3′) to verify the WT1 origin of the product. Finally, the PCR product was cloned using the pCR-Script Amp SK (+) Cloning kit (Stratagene). After screening of bacterial colonies using PCR (sense primer in intron 5, 5′-GAA CCC TGC ATC TAA AGT GG-3′ and antisense primer in exon 6, 5′-TCG TAC CCT GTG CTG TGG-3′), the positive clones were grown in 2YT broth, and the plasmid DNA was extracted by using a modified alkaline lysis procedure using PERFECT prep kit (5 Prime→3 Prime, Inc). Cycle sequencing was performed using fluorescent dye–labeled M13 forward/reverse primer kits (Perkin Elmer). The sequencing product was examined on an ABI 373 automated DNA sequencer (Perkin Elmer). Cycle sequencing using dye terminators (Perkin Elmer) and manual sequencing using AmpliCycle Sequencing Kit (Perkin Elmer) were also performed to confirm automated sequencer data.

## Western Immunoblotting

The cells were grown to 100% confluence, and protein was extracted as described in detail elsewhere [13]. Samples were subjected to SDS-polyacrylamide gel electrophoresis (10% gel) at 90 mA for 3 hours. Gels were transferred to polyvinylidene difluoride membrane, Immobilon (Amersham Pharmacia Biotech, Arlington Heights, IL) at 300 mA for 3 hours. Immunoblotting was performed by 1-hour incubation with 0.25 μg/mL anti-WT1 primary antibody, C19 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and subjected to secondary antibody incubation at 1:2,000 dilution. After the Tween 20-Tris-buffered saline wash, the signals were detected by enhanced chemiluminescence using the ECL+Plus detection kit (Amersham Phamacia Biotech). Signals were usually detected within 1 minute of exposure to Hyperfilm ECL (Amersham). As a specificity control, duplicate western blots were incubated in parallel with anti-WT1 antibody preincubated with the immunizing peptide supplied by the manufacturer (Santa Cruz Biotechnology).

## DNA Extraction and Southern Hybridization Analyses

Approximately 5 million cells were subjected to DNA extraction [21]. Protein was digested with proteinase K at a final concentration of 0.5 mg/mL, and RNA was eliminated with RNase A at a final concentration of 0.5 mg/mL by treatment at 55°C for 3 hours. The DNA was then isolated by phenol chloroform extraction. The high-molecular-weight DNA was recovered with 3.5 M ammonium acetate and 2.5-volume 100% cold ethanol. The DNA was dried and dissolved in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Ten micrograms DNA was then digested with *Eco*R I restriction enzyme overnight and subjected to 1% agarose gel electrophoresis at 40 V for 18 hours. The gel was depurinated by soaking in 0.26 M HCl for 25 minutes and then denatured with 1.5 M NaCl and 0.5 M NaOH for 30 minutes. DNA was transferred to a nylon membrane by Southern transfer and fixed onto the membrane by baking at 80°C for 30 minutes under vacuum. Hybridization was performed with two radioactive RNA probes that span exons 1 through 5 and 6 through 10 of WT1 cDNA (no. 15 and 17 in Table 1) at a concentration of $2 \times 10^6$ cpm/mL probe at 54°C for 18 hours. The autoradiograph was exposed for 24 to 72 hours.

## DNA Sequencing of Truncated WT1 Transcript

To sequence the 3′ end from exons 6 through 10 of the *WT1* gene, two overlapping RT-PCR products were generated from the M12 cell line by using two sets of primers, listed in Table 1 (numbers 17 and 18). The PCR products were run on 1.5% low-melting agarose, and the desired bands were excised and digested with β-agarase I at 42°C for 2 hours at a concentration of 50 U/mL. The DNA was then transferred to a Centricon-50 (Millipore), in which 2 mL 20% isopropanol had previously been added. Centrifugation was performed at 1,000*g* for 35 minutes. The second round of purification was performed by adding 2 mL HPLC water into the same column, followed by centrifugation at 1,000*g* for 35

minutes. The purified PCR products were then cloned into pCR-Script Amp SK (+) plasmid (Strategene). The bacterial colonies were grown in 2YT broth, and the plasmids were extracted by modified alkaline lysis method using PERFECT prep kit (5 Prime→3 Prime, Inc). After selection by restriction enzyme digestion, the positive clones were sequenced on a 373 automated DNA sequencer (Perkin Elmer) using fluorescent dye–labeled M13 forward/reverse primer kits (Perkin Elmer). Each sequence was determined on both strands.

## Results

This report shows three significant and novel findings concerning *WT1* gene expression. (1) *WT1* expression in PC-3 showed the expected normal-size (3.1-kb) transcript, whereas the cell lines comprising sublines of a human SV40 large T-antigen–immortalized prostate cancer cell lines produced two shorter transcripts (2.1 and 2.8 kb). The human breast cancer cell line, MCF7, and the leukemia cell line, K562, also produced the short transcripts detected by RT-PCR. (2) Cloning and sequencing of the 2.1-kb transcript indicated the presence of exons 6 though 10, spliced to a portion of intron 5 at the 5′ end of the truncated transcripts. (3) This 2.1-kb transcript was detected in blood samples from patients with acute leukemia (two of two samples) but not in normal peripheral-blood mononuclear cells (Fig. 1).

As shown in Fig. 1A, RT-PCR products of all cell lines prepared with primers spanning exons 8 through 10 hybridized with the 3′ end oligonucleotide probe. In contrast, we were unable to amplify a *WT1* product using primers spanning exons 1 and 2 (Fig. 1B), except in PC-3 and K562 cells (Fig. 1B).

This dilemma was resolved by a combination of northern blotting and 5′ RACE. In northern blot studies, the P69SV40TAg cells and their sublines showed two truncated WT1 transcripts of sizes 2.10 ± 0.14 and 2.84 ± 0.11 kb (Fig. 2A), whereas PC-3 cells produced the expected full-length 3.1-kb WT1 transcript (Fig. 2B). When normalized for loading (Fig. 2C), the tumorigenic M2205 and M2182 and the metastatic M12 cells produced greater amounts of the short transcripts than the parental immortalized P69SV40TAg cell line. To determine the sequence(s) at the 5′ end of the truncated transcripts, mRNA from M12 cells was examined by 5′ RACE, and the resulting products were cloned and sequenced. Figure 3C shows our findings in two independent studies. Both studies showed the presence of a portion of intron 5 at the 5′ end. Interestingly, the sequencing data (Fig. 3A, B) showed the absence of intron 5 nucleotides −101 through −1 in the WT1 transcript of this cell line. These were presumably spliced out of the primary transcript because no evidence for structural alteration of the *WT1* gene was noted by Southern blot analysis. The remaining sequence of the truncated WT1 transcripts was verified to be normal by creating, then cloning and sequencing, two overlapping cDNAs. The first extended between intron 5 and exon 7, and the second between exon 7 and the last translated codon in exon 10 (primers listed in Table 1; data not shown). Sequencing of the cloned 5′ RACE product showed 255 bp of intron 5. Taken together with the value of 1,843 bp for the size of the WT1 transcript from the beginning of exon 6 through the poly A tail, our data account for a transcript of 2,098 bp.

Western immunoblotting with anti-WT1 antibody C19, which recognizes the COOH terminus of WT1 protein, detected two small proteins (23 and 31 kDa) as immunodominant bands (Fig. 4) in P69, M12, and MCF7 cells. K562 cells expressed the expected 54-kDa normal-sized WT1 protein as the immunodominant band, as well as the smaller proteins. Both these peptides are too large to be translated from the 2.1-kb truncated *WT1* if the translation initiation codon were restricted to AUG. However, WT1 [22], as well as some other proteins [23,24], has been documented to use an alternative translation initiation codon, CUG. Use of the in-frame CUG codon in the 5′ intron 5 sequence of the truncated WT1 transcript accounts for a peptide of 201 amino acids with a molecular weight of 23.7 kDa. Although these data do not prove that the 23-kDa peptide detected in the western blot is a translation product from the short 2.1-kb WT1 transcript, they provide evidence in support of this possibility. Proteins of 54, 23, and 31 kDa were not detected with C19 antibody preincubated with the immunizing peptide.

Because of the presence of the intron 5 sequence at the 5′ end of the truncated transcript, together with the absence of 101 bases from the juncture between exon 6 and the 5′ end of this transcript, we investigated the possibility of using PCR with primers that spanned this region of the transcript to detect its presence (Table 1, no. 18). Samples of total

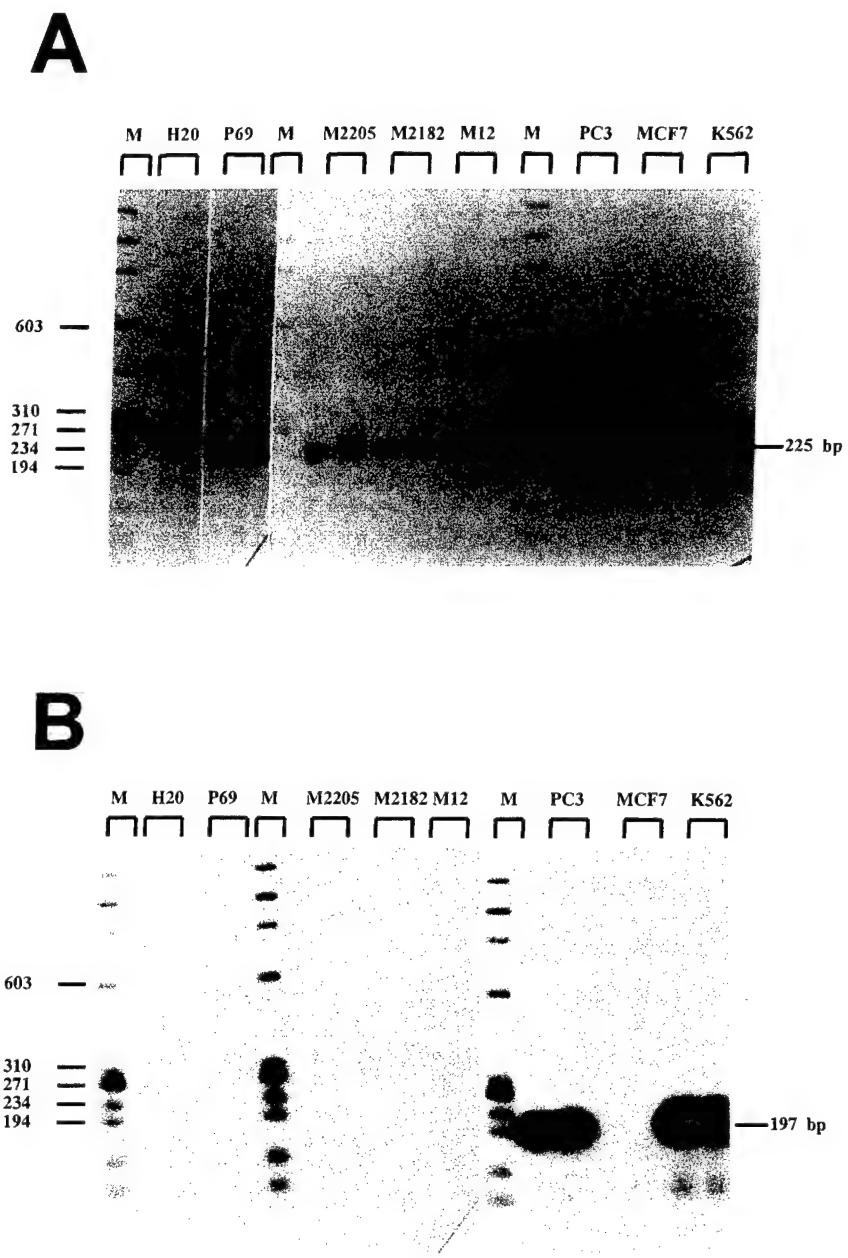



**Fig. 1.** Comparison of WT1 transcripts identified in P69SV40TAg and its sublines, PC-3, MCF7, and K562, by RT-PCR. (A) Primers span exons 8 through 10 of *WT1*, listed in Table 1. Thirty microliters reaction product in a total volume of 100 μL was loaded per lane. The products were transferred and hybridized with a probe complementary to exon 10 (Table 1). All cell lines, including P69SV40TAg and its sublines, produced the expected PCR product of 225 bp. The experiments were performed a minimum of three times. (B) Primers span exons 1 through 2 of *WT1* (Table 1). After RT-PCR, the products were transferred and hybridized with a probe complementary to the 3′ end of exon 1 (Table 1). Note the presence of WT1 transcript in PC-3 and positive control K562 but its absence from P69SV40TAg and its sublines, as well as MCF7. The experiments were performed a minimum of three times. Lanes identified as follows: M ⇒ markers; H20 ⇒ negative control, water only; cell lines (source of RNA) indicated at top. RNA sample from each line run in duplicate, indicated by brackets.

RNA obtained from the P69SV40TAg cell lines and MCF7 cells, which had been treated twice with DNase, as well as total RNA treated once with DNase obtained from normal mononuclear cells, K562 cells and samples of peripheral-blood leukocytes from a patient with acute myelocytic leukemia and from a patient with acute lymphocytic leukemia were examined (Fig. 1). In samples subjected to two treatments with DNase that contained the truncated transcript, an expected 121-bp fragment was detected. In each of the samples treated only once with DNase, a 222-bp fragment consistent with amplification of genomic DNA was observed, including the sample extracted from peripheral-blood mononuclear cells. However, the 121-bp fragment reflecting the presence of the truncated transcript was not seen in the normal peripheral-blood mononuclear cells but was observed in the K562 cell line and in two samples of peripheral-blood cells from a patient with acute myelocytic leukemia and another with acute lymphocytic leukemia.

## Discussion

In this report, detection of a novel 2.1-kb WT1 transcript was shown in a new model of prostate

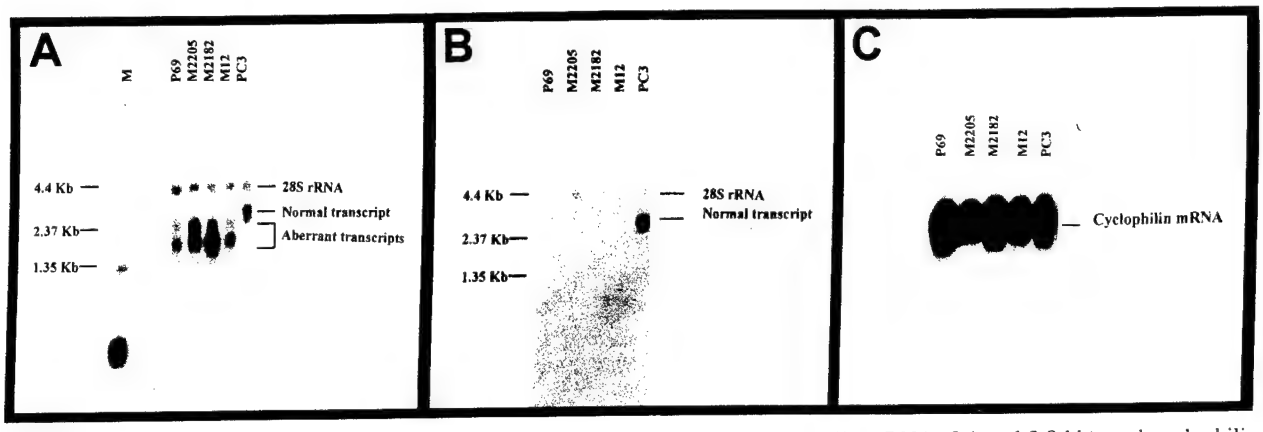



**Fig. 2.** Northern blot analysis of expression of expected 3.1-kb WT1 mRNA, novel WT1 mRNA (2.1 and 2.8 kb), and cyclophilin mRNA (loading control). Analysis conducted in three separate experiments. (A) Northern blot hybridized with probe spanning 3′ end of WT1 (exons 7 through 10; Table 1). Expected 3.1-kb transcript detected only from PC-3. Novel short transcript detected at 2.1 and 2.8 kb in P69SV40TAg and its sublines, but absent from PC-3 cells. (B) Northern blot hybridized with probe spanning 5′ end of WT1 (exons 1 through 5; Table 1) shows strong hybridization signal only from PC-3 cells. (C) Sample loading was normalized by comparison with stripped blot reprobed with riboprobe complementary to cyclophilin mRNA (template provided by Ambion, Inc). Lanes in A, B, C: M ⇒ markers (kb); source of mRNA identified by label at top of each lane. Note: C is at greater magnification than A and B.

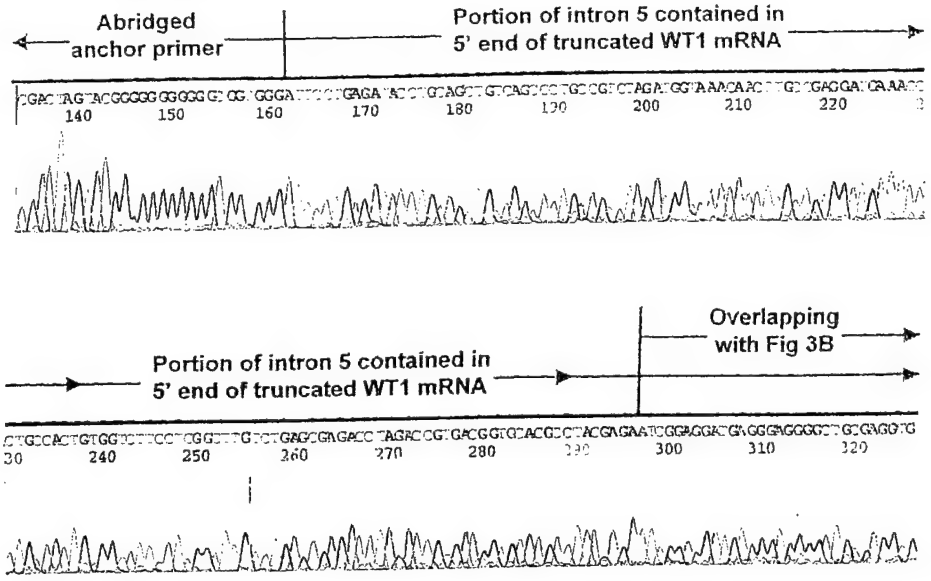
cancer progression. By using RT-PCR, this transcript was also detected in the leukemia cell line, K562, and breast cancer cell line, MCF7, as well as peripheral blood from two patients with acute leukemia, but not in normal peripheral-blood mononuclear cells. Southern blot analysis of the *WT1* gene did not show structural modifications for the cell lines that expressed the truncated transcript. The absence of intron 5 nucleotides −101 to −1 in the 5′ end of the truncated transcript is postulated at this juncture to be caused by splicing. The donor site for such a splicing event would be TCT/GTGAGC. Although we have not identified a specific instance in which this sequence has been reported to be a splice donor sequence, it shares 5/9 homology with the traditional consensus sequence (A or C)AG/GT(A or G)AGT and 5/6 homology with the variant/GTGAGT reported by Ohshima and Gotoh [25]. Both the absence of evidence of WT1 exon 1 in the 2.1-kb truncated transcript and that sequencing data indicate a start site in intron 5 raise the possibility that this transcript is initiated at an ectopic promoter in intron 5. However, we cannot be certain we have cloned the entire 5′ end of the 2.1-kb truncated transcript; therefore, the possibility that it arises from some form of alternate splicing cannot be excluded.

The origin and function of the 2.8-kb truncated fragment is unclear. A 2.5- to 2.8-kb WT1 transcript has been reported earlier in testis [26] and mesothelial cells [27], but no studies were performed to identify its sequence, structure, or function. All clones isolated in the 5′ RACE studies showed the presence of intron 5 sequence, as well as deletion of intron 5 nucleotides −101 to −1. This observation, and that the 2.8-kb transcript seemed to hybridize well to the 414-bp 3′ WT1 probe but not to the 5′ WT1 probe, suggests that the two truncated transcripts share structural similarity. However, additional studies are required to clarify this relationship.

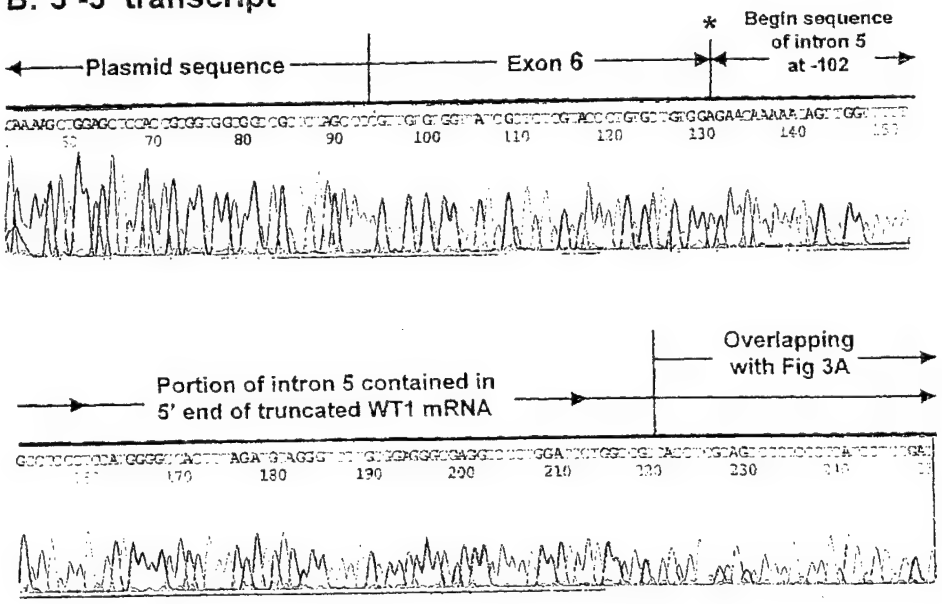
Although mutations of *WT1* have been described in Wilms' tumors [28], mesotheliomas [29], leukemias [30], Denys-Drash syndrome [31], and Frasier

**Fig. 3.** The PCR product obtained from the 5′ RACE procedure performed on mRNA from M12 cells was cloned and sequenced. Automated cycle sequencing by (A) M13 forward and (B) M13 reverse primer identified a portion of intron 5 sequence located at the 5′ end of the transcript beginning 356 bp upstream from exon 6. Note that the 101-bp portion of intron 5 from −101 to −1 was spliced out to generate the mature mRNA. The 5′ RACE procedure was repeated twice and sequencing performed three times, with the same result. (C) Schematic diagram of 2.1 kb of truncated WT1 mRNA in human prostate cell lines. The transcript appears to consist of 255 bp of intron 5 plus exons 6 through 10 of WT1. A 101-bp portion of intron 5 has been spliced out to make a mature mRNA. The resultant WT1 transcript is approximately 2,000 bp, similar to the size of the smaller truncated transcript detected by northern blot (Fig. 2A). Note that the new splice donor site shows partially preserved consensus sequence.

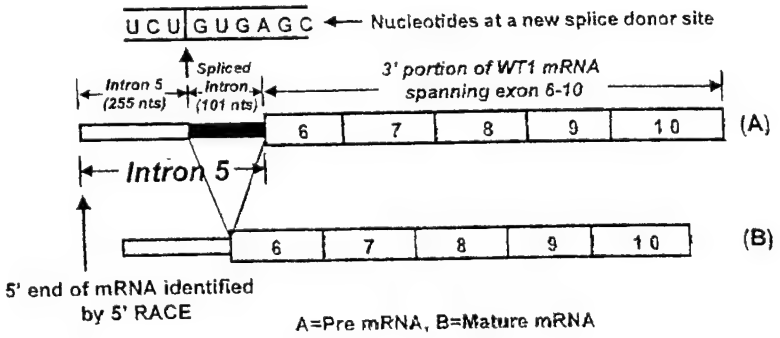
A: 5'-3' transcript




B: 3'-5' transcript




C



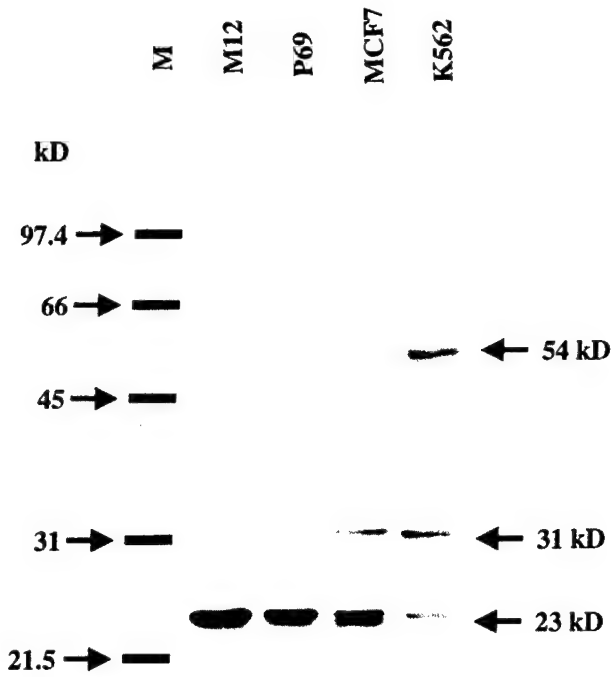



**Fig. 4.** Expression of WT1 protein detected by western immunoblotting in human prostate cancer cell lines, P69, and M12; breast cancer cell line, MCF7; and leukemia cell line, K562. Total protein extracted as described in Materials and Methods was subjected to SDS-polyacrylamide gel electrophoresis (10%), western blotted, and exposed to antibody C19, which recognizes the COOH terminus of WT1. The immunodominant bands in P69, M12, and MCF7 migrated at 31 and 23 kDa, but the expected 52- to 54-kDa WT1 protein was absent. The positive control, K562, expressed the normal 54-kDa protein, as well as the shorter forms. These experiments were performed a minimum of three times. Migration of molecular-weight marker indicated by number on left (kDa). Source of protein indicated by names of cell lines at the top of the lanes.

syndrome [32], most involve insertions or point mutations in exon 1, 7, or 9 with loss of all or part of the zinc-finger region. Splice-donor site mutations in intron 9 have been reported in cases of Frasier syndrome [32].

To our knowledge, this report describes the first demonstration of this 2.1-kb WT1 transcript with an intron 5 sequence at its 5′ end. It is noteworthy that the production of this novel, short transcript occurs in both human prostate cell lines generated by immortalization with the SV40 large T-antigen gene and the human breast cancer cell line, MCF7, which was derived originally from a spontaneous breast cancer, and also K562, an acute leukemia cell line. Using primers in intron 5 and exon 6, we detected this novel transcript in blood samples from acute leukemia cases (two of two cases) by RT-PCR but not in normal peripheral-blood mononuclear cells, suggesting its use as a tumor marker. A useful feature derived from the structure of the 2.1-kb truncated transcript is that RT-PCR produces a signal specific for the transcript that can be easily distinguished from DNA contamination. The latter is possible because the primers span the portion of the transcript showing the 101-bp deletion from nucleotides −101 to −1. The resultant PCR product is shorter than that generated by contaminating DNA. This provides a sensitive method for detecting this 2.1-kb WT1 transcript in spontaneous primary human cancers, as well as cancer cell lines.

The mechanism(s) by which the 2.1- and 2.8-kb WT1 transcripts might contribute to tumor cell ag-

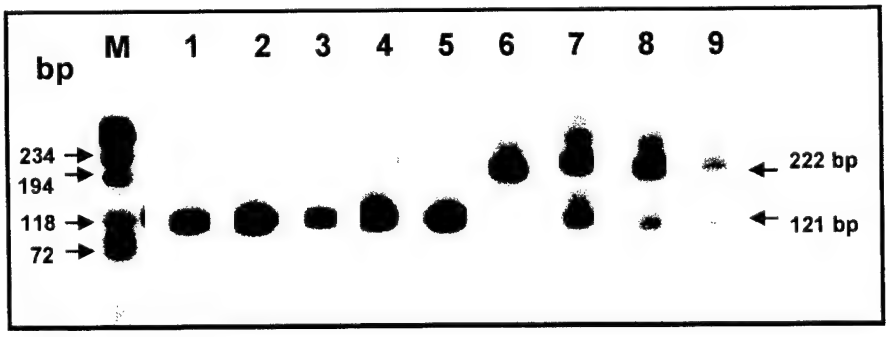



**Fig. 5.** RT-PCR product using primers in intron 5 and exon 6 (no. 18, Table 1). Total RNA was obtained from P69SV40TAg cell lines, K562, MCF7, blood samples from patients with acute leukemia, and normal peripheral-blood mononuclear cells. Samples from the P69SV40T cell lines and MCF7 samples were subjected to two treatments with DNase, whereas the RNA from the remaining samples was treated only once with DNase. Thirty microliters reaction product was examined on 1.5% agarose gel, transferred to nylon membrane, and probed with an oligonucleotide in exon 6 (Table 1; no. 11; sense oligonucleotide). The 222-bp fragment represents amplified product from contaminating DNA. The 121-bp fragment (arrow) represents product from truncated transcript in which a 101-bp segment of intron 5 between −101 and −1 has been removed by splicing. Lanes identified as follows: 1 ⇒ P69; 2 ⇒ M2205; 3 ⇒ M2182; 4 ⇒ M12; 5 ⇒ MCF7; 6 ⇒ normal peripheral-blood mononuclear cells; 7 ⇒ K562; 8 ⇒ peripheral-blood mononuclear cells from patient with acute myelocytic leukemia; 9 ⇒ peripheral-blood mononuclear cells from patient with acute lymphocytic leukemia.

gressiveness is unclear at present. However, the sequence analysis (illustrated schematically in Fig. 3C) shows the absence of most of the transactivation region of *WT1*. The lack of the transactivation domain is likely to alter the functional consequences of increased production of this *WT1* gene product. Given the documented effect of interaction with other proteins on the biological consequence of *WT1* expression, the protein encoded by these short transcripts may contribute directly and/or indirectly to tumorigenic and/or metastatic capacity in a novel way. Survey of expression of this transcript in benign and malignant prostate glands, as well as other human cancers, is ongoing.

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# Transcriptional Regulation of Insulin-Like Growth Factor-I Receptor Gene Expression in Prostate Cancer Cells*

SUSAN E. DAMON, STEPHEN R. PLYMATE, JULIE M. CARROLL, CYNTHIA C. SPRENGER, CHEVABOON DECHSUKHUM, JOY L. WARE, AND CHARLES T. ROBERTS, JR.

*Geriatric Research Education and Clinical Center, Veterans Administration Puget Sound Health Care System (S.E.D., C.C.S., S.R.P.), Tacoma, Washington 98493; Department of Medicine, University of Washington (S.E.D., S.R.P.), Seattle, Washington 98195; Department of Pathology, Medical College of Virginia/Virginia Commonwealth University (C.D., J.L.W.), Richmond, Virginia 23298; and Department of Pediatrics, Oregon Health Sciences University (J.M.C., C.T.R.), Portland, Oregon 97201*

**ABSTRACT**

A marked decrease in the type 1 insulin-like growth factor (IGF) receptor (IGF-IR) occurs in prostate epithelial cells during transformation from the benign to the metastatic state. One of the principal regulators of IGF-IR gene expression, the WT1 tumor suppressor, is expressed in prostate cancer and in prostate cancer cell lines. The purpose of this study was to determine whether the decrease in IGF-IR expression was transcriptionally regulated, and whether WT1 action may be involved in the repression of the IGF-IR gene in prostate cancer cells. The P69 cell line was derived by immortalization of human primary prostate epithelial cells with simian virus-40 T antigen and is rarely tumorigenic. The M12 line was derived from the P69 line by selection for tumor formation in nude mice and is tumorigeneic and metastatic. P69 cells express 20,000 IGF-IR/cell, whereas M12 cells express 3,500 IGF-IR/cell. These differences in receptor number are reflected in proportional differences in IGF-IR mRNA levels. To assess IGF-IR promoter activity in these cell lines, each was transiently transfected with luciferase reporter vectors containing the IGF-IR gene transcription start site and 476 bp of 5′-flanking sequence, 640 bp of 5′-untranslated region sequence, or both regions. The promoter activity of the full-length construct was 50% lower ($P < 0.01$) in M12 cells compared with P69 cells, the activity of the 5′-flanking region construct was 53% lower ($P < 0.0001$), and that of the 5′-untranslated region construct was 36% lower ($P = 0.01$). P69 clones stably transfected with a WT1 expression vector exhibited decreased expression of the endogenous IGF-IR gene and decreased promoter activity in transient transfection assays with IGF-IR promoter constructs containing multiple WT1 binding sites. The observed reduction in endogenous IGF-IR expression was sufficient to inhibit IGF-I-stimulated cell proliferation. These data suggest that most of the decreased expression of the IGF-IR seen in malignant prostate epithelium is the result of transcriptional repression of the IGF-IR gene, and that this repression may be due in part to the increased expression of the WT1 tumor suppressor in metastatic prostate cancer. (*Endocrinology* **142:** 21–27, 2001)

THE INSULIN-LIKE growth factor (IGF) receptor (IGF-IR) mediates the mitogenic, transforming, differentiating, and antiapoptotic effects of the IGF ligands, IGF-I and IGF-II (1–3). Transformation of the prostate epithelium is a multistep process that is initially IGF-IR dependent, but as tumorigenicity increases and the cancer becomes androgen independent and metastatic, IGF-IR expression decreases (4–6). Despite this reduction in IGF-IR as prostate epithelial cells (PEC) become more aggressive, the IGF-IR appears to continue to play a role in tumor growth. The use of IGF-IR antisense or dominant negative mutants that further reduce IGF-IR activity in prostate cancer cells results in decreased tumor invasiveness *in vitro* and decreased tumor formation in nude mice (7, 8). These data indicate that although markedly decreased in number, the IGF-IR retains its antiapoptotic and proliferative functions and contributes to the maintenance of the malignant phenotype.

The P69SV40T series of PEC has been previously described with respect to tumor formation and the IGF system (9–11). The parental P69 cell line is poorly tumorigenic and not metastatic, whereas the M12 subline is greater than 90% tumorigenic and is metastatic (11). P69 cells express levels of IGF-IR similar to those seen in primary PEC. IGF-IR expression decreases significantly in the metastatic M12 subline, as has been reported in human metastatic prostate cancer and in murine prostate cancer models (5, 6, 10). The P69/M12 cell system thus represents an appropriate model in which to study the role and the regulation of the IGF-IR in the progression of prostate cancer.

When the IGF-IR is reexpressed in the malignant, metastatic M12 cell line to levels found in the benign parental P69 cell line, there is a concomitant decrease in tumor growth and metastasis *in vivo*, decreased growth in soft agar, and an increased sensitivity to apoptosis *in vitro* (12, 13). These data suggest that normal IGF-IR levels are necessary to maintain a less aggressive phenotype, and that altered responsiveness

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Address all correspondence and requests for reprints to: Dr. Charles T. Roberts, Department of Pediatrics (NRC-5), Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201. E-mail: robertsc@ohsu.edu.
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to IGF-I is alone sufficient to modulate the malignant phenotype.

The observation that both IGF-IR mRNA and protein decrease significantly in the transition from benign to malignant human prostate epithelium *in vivo* and *in vitro* is consistent with the idea that this decrease is due to transcriptional regulation (4). The transcription of the IGF-IR gene is controlled by various factors, including Sp1 (14), p53 (15, 16), and WT1 (17–20). The WT1 gene is located at 11p13 and encodes a DNA-binding protein with four $C_2$-$H_2$ zinc finger domains (21, 22). Multiple versions of the WT1 protein are generated as a result of alternative splicing, RNA editing, and alternative translation initiation codons (23–25). WT1 variants produced as a result of the alternative splicing of exon 9 are designated WT1(+KTS) or WT1(−KTS) depending on the presence or absence of a three-amino acid (Lys, Thr, Ser) insert between zinc fingers 3 and 4. The WT1 proteins arising from these transcripts appear to have altered specificity for DNA binding and may regulate transcription in different ways (26–28). The WT1 gene product belongs to the early growth response family (29) and represses the transcription of several growth factor and growth factor receptor genes, including the IGF-IR (17–20, 30). The IGF-IR promoter contains 12 WT1 DNA-binding sites (18) in the proximal 5′-flanking and 5′-untranslated regions (5′-UTRs; Fig. 1) that contribute in an additive manner to WT1 repression of IGF-IR promoter activity.

There is mounting evidence that suggests WT1 may play an important role in prostate pathophysiology. We have previously demonstrated that WT1 expression is decreased, whereas IGF-IR and IGF-II expression is increased, in stromal cells from patients with benign prostatic hyperplasia (31). Ng *et al.* reported that WT1 protein increases commensurate with the Gleason score in archival prostate needle biopsy specimens (32). Finally, WT1 message levels increase dramatically in several malignant, metastatic PEC lines and human prostate carcinomas compared with either human benign prostate epithelium or nonmetastatic prostate cell lines, and the proportion of WT1(+KTS) *vs.* WT1(−KTS) transcription splice variants varies between nonmetastatic (1:1) and metastatic (2:1) prostate cancer cell lines (33).

In this study we have examined the level at which IGF-IR expression is regulated in metastatic and nonmetastatic prostate cancer cells and the potential contribution of WT1 to this regulation. We measured IGF-IR promoter activity in the benign P69 cell line (which expresses high levels of IGF-IR) and the malignant, metastatic M12 subline (which expresses low levels of IGF-IR) using IGF-IR promoter fragments to drive the expression of a firefly luciferase reporter gene. We also analyzed IGF-IR promoter activity and IGF-I-stimulated cell proliferation in stably transfected P69 cells overexpressing WT1(+KTS) to determine whether an increase in WT1 expression could influence IGF-IR expression and IGF responsiveness.

## Materials and Methods

### *Materials*

RPMI 1640, epidermal growth factor (EGF), and dexamethasone were purchased from Sigma (St. Louis, MO). Gentamycin, fungizone, and geneticin (G418) were obtained from Life Technologies, Inc. (Grand Island, NY). FBS was purchased from HyClone Laboratories, Inc. (Logan, UT). Insulin, transferrin, and selenium were purchased as the additive ITS from Sigma. PrEBM and supplements for growth of primary human PEC were obtained from Clonetics (San Diego, CA). The pGL3 control and enhancer plasmids, the luciferase assay system with reporter lysis buffer, Aqueous$_{\text{one}}$ 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide cell proliferation kits, and Tfx-50 were obtained from Promega Corp. (Madison, WI). WT1 (C-19) polyclonal and IGF-IR polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). RNA-STAT60 was purchased from Tel-Test (Friendswood, TX).

### *Cell lines and culture*

Derivation of the P69SV40T (P69), and M12 cell lines has been previously described (9–11). Briefly, human PEC were immortalized with simian virus-40 T antigen to produce the immortalized P69 cell line. P69 cells were injected sc into athymic nude mice and produced tumor nodules in 2 of 18 animals after 180 days. These nodules were reimplanted into athymic nude mice and after 3 passages resulted in M12 cells, which demonstrated a short latency period of 7–10 days to tumor formation in all 10 animals receiving sc injection and which were locally invasive and metastatic when injected orthotopically (11). All cells were cultured in RPMI 1640 medium supplemented with 10 ng/ml EGF, 0.1 mM dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium, fungizone, and gentamicin (ITS medium) at 37 C in 5% $CO_2$. PC-3 and LNCaP cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% FBS. Human primary PEC at passage 4 were

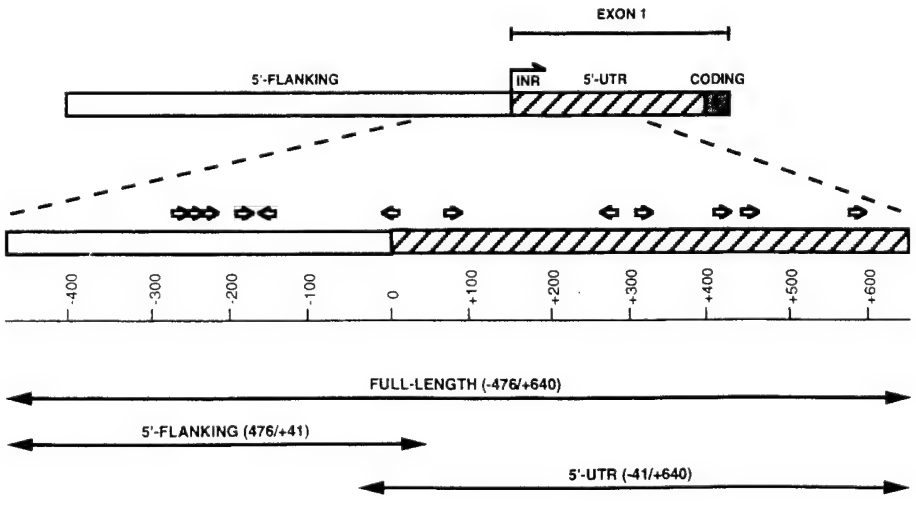


FIG. 1. Schematic representation of the IGF-IR proximal promoter region, exon 1, and the sequences incorporated into the luciferase reporter constructs used in transient transfection assays. *Arrows* depict the location and orientation of WT1-binding sites (consensus; 5′-GXGGGGGXG-3′) as defined by gel shift and deoxyribonuclease I footprinting analyses (18). INR, The initiator motif directing specific transcription initiation from this TATA-less promoter.

obtained from Clonetics and seeded at $2.5 \times 10^3$ cells/T-25 flask in PrEBM supplemented with bovine pituitary extract, insulin, hydrocortisone, GA-1000, retinoic acid, transferrin, $T_3$, epinephrine, and human EGF supplied in a Bulletpack (Clonetics).

The B7 and D16 cell lines are stable clones of P69 cells transfected with a 3.0-kb human WT1(+KTS) cDNA cloned into the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA) (20). Clones were cultured in RPMI 1640 medium supplemented as described above and with 200 μg/ml G418. Transfectants were characterized for the expression of WT1 by RT-PCR and Western immunoblot, IGF-IR expression by Western immunoblot, and IGF-I-induced proliferation by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay.

### *Ribonuclease (RNase) protection assay of IGF-IR mRNA levels*

Primary human PEC, P69, M12, LNCaP, and PC3 cells were harvested at 80% confluence, and total RNA was prepared using RNA-STAT60. RNA quantity and integrity were verified by electrophoresis of 2-μg aliquots on ethidium bromide-stained 1% formaldehyde-agarose gels, followed by densitometric analysis using a Gel-Doc 1000 system and Molecular Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA). Twenty-microgram aliquots of total RNA were used in a RNase protection assay with a [$^{32}$P]UTP-labeled antisense RNA probe complimentary to exon 2 and 3 sequences of the human IGF-IR mRNA as previously described (17). Hybridization signals were quantitated using a GS 375 phosphorimager (Bio-Rad Laboratories, Inc.).

### *Cell surface IGF-IR levels*

IGF-IR number per cell was determined by Scatchard analysis as previously described (10). Briefly, cells plated in 24-well culture dishes were incubated in duplicate with [$^{125}$I]IGF-I (Amersham Pharmacia Biotech, Arlington Heights, IL) for 3 h at 26 C at concentrations ranging from 0.05–2 nM. Nonspecific binding was determined by adding a 500-fold molar excess of unlabeled IGF-I (a gift from Eli Lilly & Co., Indianapolis, IN). After incubation, the cells were placed at 4 C for 15 min and rinsed with ice-cold PBS buffer containing 1% BSA. Cells were solubilized in 0.2 N NaOH, and the cell-associated radioactivity was counted in a γ-counter. Protein was measured using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL), and cell number was determined by counting cells in replicate wells. The maximum binding capacity and apparent dissociation constant were determined using Scatchard analyses as previously described (10). Each experiment was performed in triplicate.

### *RT-PCR of WT1 mRNA*

Total RNA was extracted from 20,000 cells using the Ultraspec reagent and extraction protocol (Biotex Laboratories, Inc., Houston, TX). Total RNA was dissolved in diethylpyrocarbonate-treated water containing RNasin (1 U/μl; Promega Corp.) and stored at −80 C. One microgram of total RNA was used in the RT reaction with 2 μM antisense primer (5′-TCAAAGCGCCAGCTGGAGTTT-3′; corresponds to exon 10 of the WT1 gene), 75 mM KCl, 50 mM Tris (pH 8.3), 3.0 mM $MgCl_2$, 0.8 mM deoxy-NTPs, and 4.2 U/μl Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) for 1 h at 42 C followed by heat inactivation at 95 C for 5 min. One tenth of the first strand reaction was used in the amplification reaction. PCR was performed in 56 mM KCl, 19.6 mM Tris (pH 8.3), 1.6 mM $MgCl_2$, and 0.2 mM deoxy-NTPs. The sense primer (5′-AGACATACAGGTGTGAAACC-3′; corresponds to WT1 exon 8 sequence) and the antisense primer described above were used at a final concentration of 1 μM and produced a fragment of 225 bp. Forty cycles of PCR were performed using denaturation for 1 min at 95 C, annealing for 30 sec at 60 C, and extension for 1 min at 72 C using a GeneAmp 9600 thermal cycler (Perkin-Elmer Corp., Foster City CA). The PCR product was analyzed by electrophoresis through a 1.5% agarose gel and transferred to Zetaprobe (Bio-Rad Laboratories, Inc., Hercules, CA). DNA was immobilized to the membrane with a UV cross-linker (Stratagene, La Jolla, CA) and hybridized with the sense primer that had been end-labeled with [γ-$^{32}$P]ATP (3,000 Ci/mmol; NEN Life Science Products, Boston, MA) at 55 C for 16 h. Autoradiographs were developed after a 48-h exposure, and hybridizing bands were quantitated by densitometry.

### *IGF-IR promoter reporter constructs*

Transient transfection experiments used the following rat IGF-IR gene promoter fragments ligated upstream of the firefly luciferase reporter cDNA in pGL3 vectors: 1) fragment −476 to +641, designated the full-length IGF-IR proximal promoter (20); 2) fragment −476 to +41, designated the 5′-flanking region of the IGF-IR and generated by PCR using a forward (5′-GGGCTAGCTTTTTCACAGAGCGGGCCAG-3′) and reverse (5′-GGAAGCTTCAAGAACCTCAGCCTCACCG-3′) primer; and 3) fragment +41 to +640, designated the 5′-UTR, generated by PCR using a forward (5′-GGGCTAGCACGTGTGCGCGGC-CCCGAGA-3′) and reverse (5′-GGAAGCTTCAGAAAGAGGAG-CAAAGCCC-3′) primer. Nucleotide +1 corresponds to the transcription initiation site. All PCR primers were used at 5 μM. The forward primers of the 5′-flanking and 5′-UTR fragments contained a *Nhe*I site, and the reverse primers contained a *Hin*dIII site. Thirty-five cycles of PCR were performed using a Gradient Cycler 40 (Stratagene). The PCR-generated fragments were cut with *Nhe*I and *Hin*dIII and ligated into the pGL3 reporter plasmid. Constructs were verified by automated fluorescent sequencing. These constructs are schematized in Fig. 1.

### *Transient transfections*

P69, M12, D16, and B7 cells were transfected with the various IGF-IR promoter/luciferase vectors using Tfx-50 according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates in complete RPMI medium containing 5% FCS and grown to 60% confluence. Each well received 1.5 μg vector DNA using a 3:1 charge ratio of Tfx-50/DNA in RPMI. After 1 h, the medium was replaced with serum-containing medium, and the plates were incubated for 48 h, at which time the cells were lysed, and luciferase activities were measured. All cells used in these experiments were *Mycoplasma* free as determined by PCR with a *Mycoplasma* PCR primer set (Stratagene).

### *Luciferase assays*

Luciferase activity was determined using the Luciferase Assay System (Promega Corp.) according to the manufacturer's protocol. Cells were lysed with 1 × reporter lysis buffer and analyzed on a Nichols Institute Diagnostics Luminometer 400 (San Juan Capistrano, CA). Each sample was read for 20 sec. Transfection efficiency was determined by transfection of duplicate wells with the pGL3 control vector in which luciferase expression is driven by the simian virus 40 promoter as well as cotransfection with a pCMV β-galactosidase reporter plasmid as previously described (20).

### *Western immunoblots*

Cells were collected for Western blot analysis by washing with PBS and 0.1% BSA. Cells were lysed in 100 μl SDS sample buffer (10% glycerol, 2% SDS, and 0.001% bromophenol blue) and heated for 5 min at 100 C. Electrophoresis of cell lysates was performed on a 10% SDS-PAGE gel. Protein concentration was determined with the bicinchoninic acid protein assay reagent, and 50 μg protein were added to each lane. After electrophoresis, proteins were transferred to nitrocellulose by electroblotting and probed with either IGF-IR or WT1 antibodies. Bound antibody was detected using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Buffers and wash times were as suggested in the ECL kit, except that nitrocellulose was washed in 10% hydrogen peroxide for 10 min before the first blocking step.

### *Assays of tumor growth in vivo*

The tumorigenicity of P69, M12, and PC-3 cell lines was assessed by sc injection of $10^6$ cells into male athymic nude mice that were 6–8 weeks old at the time of injection. Each group of 10 mice was injected with 1 of the 3 cell lines. The percentage of tumor formation was defined as the percentage of mice injected with tumor cells that developed tumor nodules over the 12-week period postinjection. All mice were maintained in a specific pathogen-free barrier facility. *In vivo* experiments

were conducted under a protocol approved by the VAPSHCS and University of Washington animal care and use committees.

### *Cell proliferation*

Cell proliferation was determined with a tetrazolium assay for quantification of viable cells (Cell Titer 96 Aqueous kit, Promega Corp.). Twenty-five hundred cells were added to each well of a 96-well plate, and test reagents were added at the times indicated. IGF-I was added to the ITS medium at the time of plating. After 72 h in culture, the tetrazolium salt and dye solution was added, color development was allowed to proceed for 2–3 h at 37 C, and absorbance at 570 nM was measured for each well. Each cell line was tested in three separate experiments. MTS results were confirmed by cell counts. The correlation between cell number and the MTS tetrazolium assay in our laboratory is r = 0.97.

## Results

### *Relationship between tumor formation and IGF-IR expression*

When tumor formation in nude mice was compared with IGF-IR expression in a series of PEC lines, there was an inverse correlation with IGF-IR protein and mRNA levels (Fig. 2). Specifically, the M12 and PC-3 cell lines are highly tumorigenic and metastatic and display the lowest levels of IGF-IR protein and mRNA. The poorly tumorigenic P69 cell line and primary PEC express high levels of IGF-IR protein and mRNA. LNCaP cells, which are weakly metastatic, exhibit intermediate levels of IGF-IR expression. No changes in affinity for IGF-I were seen.

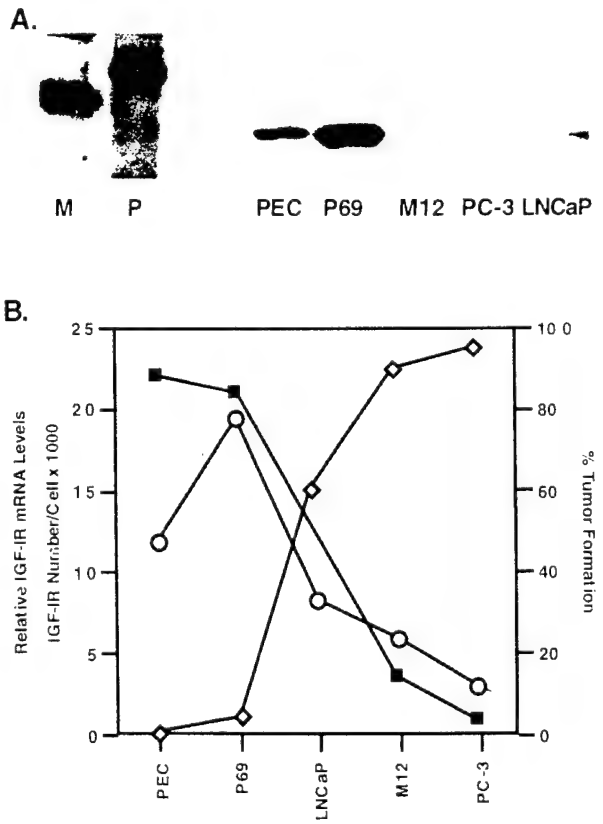


FIG. 2. A. RNase protection assay of IGF-I mRNA levels in prostate epithelial cells in primary culture and prostate epithelial cell lines. M, Molecular weight marker; P, probe. PEC, P69, LNCaP, M12, and PC-3 refer to cells and cell lines described in the text. Shown are the 307-base marker (lane M) the 394-base IGF-IR antisense RNA probe (lane P), and the 379-base protected probe band corresponding to endogenous IGF-IR mRNA in the various samples. B. Comparison of IGF-IR number per cell (*squares*), relative IGF-IR mRNA levels as determined by scanning densitometry of the autoradiograph in A (*circles*), and tumorigenicity of cell lines when implanted sc in nude mice (*diamonds*).

### *IGF-IR promoter activity in P69 and M12 cells*

We next determined whether IGF-IR promoter activity was lower in the malignant, metastatic M12 cells, which express low levels of endogenous IGF-IR, than in the poorly tumorigenic P69 cells, which express high levels of IGF-IR. IGF-IR promoter activity was assayed in P69 and M12 cells transiently transfected with three IGF-IR promoter constructs. The full-length IGF-IR construct contained a 1116-bp promoter fragment spanning bp −476 to +640 of the IGF-IR proximal promoter. The second IGF-IR construct contained the 5′-flanking region and the transcription start site (bp −476 to +41), and the third construct contained the transcription start site and the 5′-UTR (bp +41 to +640). As shown in Fig. 3, the activity of all three constructs was significantly lower in M12 cells than in P69 cells (50%, $P < 0.01$; 53%, $P < 0.0001$; and 36%, $P < 0.01$, respectively).

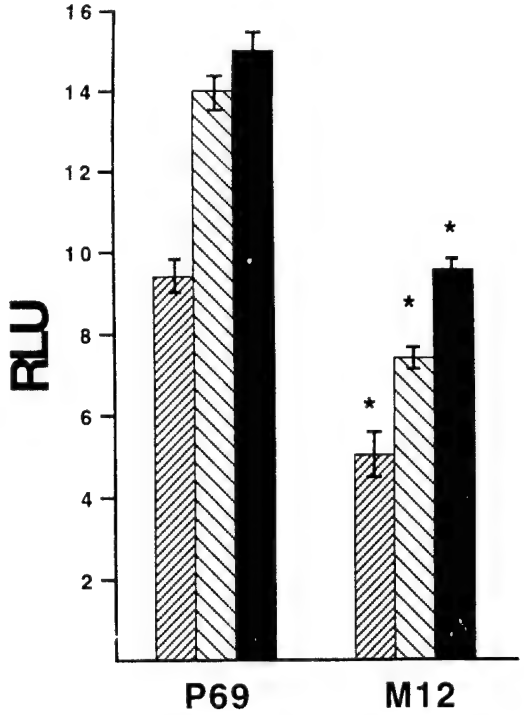


FIG. 3. IGF-IR promoter activity in P69 and M12 cells. *Dotted bars*, Luciferase activity of the full-length promoter fragment (bp −476 to −641); *cross-hatched bars*, the activity of the 5′-flanking fragment (bp −476 to −41); *solid bars*, the activity of the 5′-UTR fragment (bp +41 to −640). RLU of luciferase activity was corrected for transfection efficiency as described in *Materials and Methods*. *, $P < 0.01$ for the full-length and 5′-UTR constructs and $P < 0.0001$ for the 5′-flanking construct. M12 *vs.* P69.

*Effect of WT1 expression on IGF-IR promoter activity*

To determine the effect of increased WT1 expression on IGF-IR gene expression, IGF-IR promoter activity was analyzed in P69 cells as well as in two P69 clones that had been stably transfected with an expression vector containing a full-length WT1(+KTS) cDNA driven by the cytomegalovirus promoter. The B7 and D16 clones express WT1 mRNA at 1,901 and 5,370 copies/20,000 cells, respectively, and WT1 protein expression was confirmed by Western immunoblot (data not shown). We previously identified multiple WT1 binding sites in both the 5′-flanking region nd 5′-UTR of the IGF-IR proximal promoter and found that each region is subject to repression by WT1 in transient transfection assays (18). The activity of each of these promoter constructs was significantly lower ($P < 0.0001$) in the B7 and D16 clones compared with that in P69 cells, indicating that WT1(+KTS) suppresses IGF-IR promoter activity in these PEC (Fig. 4).

*Effect of WT1 expression on IGF-IR levels and IGF-I responsiveness*

We next determined whether WT1 suppression of IGF-IR promoter activity was sufficient to affect IGF-IR levels and IGF responsiveness in the B7 and D16 clones. IGF-IR protein levels were assessed in M12, P69, B7, and D16 cells by Western immunoblot analysis (Fig. 5). Overexpression of WT1(+KTS) resulted in a marked decrease in IGF-IR protein in the B7 and D16 clones to levels comparable to those seen in the metastatic M12 cells. The decreased level of IGF-IR in the B7 and D16 clones resulted in a decreased proliferative response to IGF-I compared with that seen in the parental P69 cell line (Fig. 6).

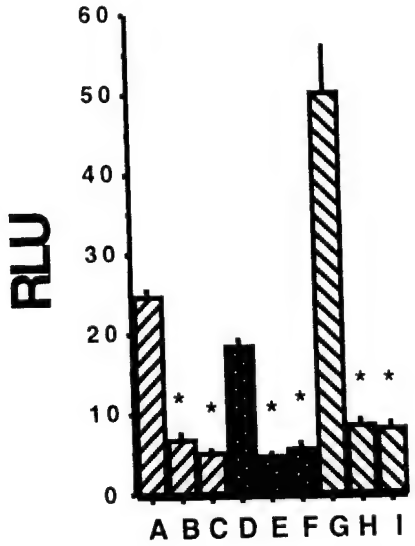


FIG. 4. IGF-IR promoter activity in P69 cells and two clonal lines derived by stable transfection of P69 with a WT1(+KTS) expression vector and designated B7 and D16. A–C, Full-length promoter; D–F, 5′-flanking regions; G–H, 5′-UTR. A, D, and G, P69 cells; B, E, and H, B7 cells; C, F, and I, D16 cells. The relative light units (RLU) of luciferase activity per mg protein was controlled for transfection efficiency as described in *Materials and Methods*. *, $P < 0.0001$ for B and C *vs.* A, for E and F *vs.* D, and for H and I *vs.* G.

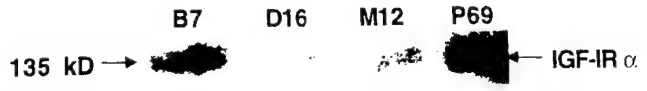


FIG. 5. Western immunoblot of M12, P69, D16, and B7 lysates probed with antibody to the α-subunit of the IGF-IR. Each lane contains 100 μg protein. Note the marked decrease in IGF-IR expression in the metastatic M12 line and the B7 and D16 clones, derived by transfection of the P69 line with a WT1 expression vector, compared with the P69 parental line.

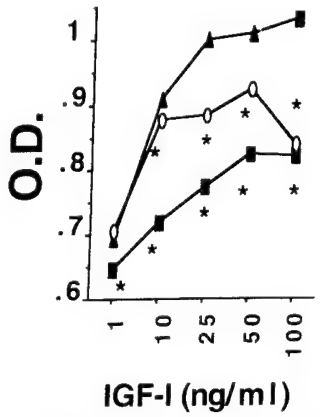


FIG. 6. Proliferation of P69, B7, and D16 cells in response to increasing concentrations of IGF-I. *, $P < 0.05$ compared with the P69 cell line at the same concentration of IGF-I. P69 cell growth is represented by *triangles*, B7 by *circles*, and D16 by *squares*.

## Discussion

The action of the IGF-IR is thought to contribute to the development of many cancers, including prostate cancer. Specifically, high circulating levels of IGF-I are a risk factor for prostate carcinoma (34, 35), and constitutive expression of IGF-I in prostate basal epithelial cells (that express high levels of IGF-IR) produces prostate cancer in transgenic mice (36). Paradoxically, a progressive decrease in IGF-IR expression is seen in human and murine metastatic disease and in the P69-M12 system employed in this study (4–6). This consistent association of decreased IGF-IR expression with the development of advanced disease may reflect the ability of IGF-I (through the IGF-IR) to exert differentiative as well as proliferative effects depending upon such factors as the complement of postreceptor mechanisms available (*i.e.* insulin receptor substrate-1) (37); resistance to such differentiative effects may be required for full acquisition of the malignant phenotype.

The coordinate decrease in IGF-IR protein or binding and mRNA levels seen in metastatic prostate cancer cells is consistent with the idea that transcriptional repression of the IGF-IR gene is a requisite for the development of advanced disease. In that case, an understanding of the molecular mechanisms governing transcription of the IGF-IR gene in PEC would provide insights into improved approaches for the treatment of refractory disease. The results of the current study demonstrate that IGF-I promoter activity itself is decreased in metastatic prostate cancer cells, and that this decrease is responsible for a substantial portion of the decreased IGF-IR mRNA and protein observed in these cells compared with their nontumorigenic or metastatic precursors. That the difference in IGF-IR mRNA levels between P69

and M12 cells is greater than the difference in promoter activity suggests that regulatory elements outside of the −476/+640 proximal promoter region analyzed in this study may influence the activity of the endogenous IGF-IR gene in the P69-M12 cell system. Alternatively, posttranscriptional regulatory mechanisms may also contribute to overall IGF-IR gene regulation in this context. Our data, however, suggest that transcriptional control is a principal component of IGF-IR gene expression in metastatic *vs.* nonmetastatic prostate cancer cells.

We have previously shown that IGF-IR gene expression is significantly increased in Wilms' tumor, and that there is a negative correlation between levels of IGF-IR mRNA and WT1 mRNA, suggesting that the IGF-IR is under the inhibitory control of the WT1 tumor suppressor protein (17). Characterization of the IGF-IR promoter using gel retardation and deoxyribonuclease I footprinting assays demonstrated 12 WT1-binding sites in the −476/+640 region of the proximal IGF-IR promoter (18). Overexpression of WT1 in Chinese hamster ovary and G401 cells resulted in decreased IGF-IR promoter activity and cell surface receptor number (18, 20).

Overexpression of WT1(+KTS) in P69 cells resulted in highly significant suppression of IGF-IR promoter activity with all three promoter constructs. Interestingly, promoter activity in the B7 clone was suppressed to the same extent as in the D16 clone, yet the levels of WT1 expression were higher in B7 cells compared with D16 cells. This may indicate that even moderate increases in WT1(+KTS) can efficiently repress IGF-IR gene expression. The effect of WT1 on IGF-IR promoter activity was paralleled by decreased expression of the endogenous IGF-IR gene and a decreased proliferative response to IGF-I. A short WT1 transcript has been recently identified in some prostate cancer cell lines that lacks the sequence derived from the first five exons (38). It remains to be determined whether the protein encoded by this novel WT1 transcript has altered binding specificity or function in prostate cells, but our previous demonstration that carboxyl-terminal versions of WT1 are functional (20) suggests that this WT1 variant may also repress IGF-IR gene expression.

In summary, this study demonstrates that the decrease in IGF-IR expression that occurs in aggressive prostate cancer appears to be primarily at a transcriptional level, and that an increase in WT1 action may constitute part of the mechanism through which IGF-IR gene expression is repressed. The level of IGF-IR expression may, in turn, affect IGF responsiveness in terms of proliferation, differentiation, or sensitivity to apoptosis. Any or all of these factors could influence the tumorigenecity of a given PEC. In light of our previous studies demonstrating decreased tumor growth and metastatic potential as a result of increased IGF-IR expression (12, 13), targeting those factors that regulate IGF-IR promoter activity may lead to novel therapies for metastatic disease.

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**Expression of WT1 Transcripts in Human Prostate Cancers**

Chavaboon Dechsukhum[1]; Carleton T. Garrett[1]; Andrea Ferreira-Gonzalez[1]; Natapol Supanatsetakul[1]; R. Tucker Burks[1,3]; William Morgan[2]; David S. Wilkinson[1], Joy L. Ware[1]*.

Department of Pathology, Medical College of Virginia Campus of Virginia Commonwealth Univeristy[1], Richmond Virginia, and Virginia Urology Center[2], Richmond, Virginia.

[3] 109 Brandon Way , Simpsonville SC 29681

*To whom correspondence should be addressed:

Joy L. Ware, Ph.D.
Box 980662
Medical college of Virginia Campus
Virginia Commonwealth University
Richmond, VA 23298
Phone: 804-828-5092
Fax: 804-828-9749
Email: jlware@hsc.vcu.edu

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Shortened title: WT1 in Human Prostate Cancers

Key words: prostate cancer, RT-PCR, WT1 and cancer

**Abstract**

**Background:** The Wilms' tumor suppressor gene (WT1) encodes a transcription factor that has an important role in genitourinary development and Wilms' tumor growth. We recently identified a novel truncated WT1 transcript in immortalized human prostate epithelial cells and their tumorigenic sublines. In this study we analyzed human microdissected benign and malignant prostate glands for expression of wild type and truncated WT1 transcripts, as well as the WT1 target genes EGFR and IGFR-1.

**Method:** RT-PCR was performed on frozen tissue (microdissected, paired, benign and malignant prostate epithelium) obtained from radical prostatectomy. Isolated total RNA was subjected to reverse transcription and amplification (RT-PCR) and probed to detect the indicated transcripts.

**Results:** Either the wild type or the truncated transcript, or both, were detected in malignant glands in 18/32 malignant while these transcripts were found in benign glands in only 10 of 32 cases. No correlation between expression of either transcript and Gleason score or preoperative serum PSA value was found. Malignant glands expressing wild type WT1 continued to express IGFR-1 and EGFR transcripts.

**Conclusion:** These findings suggest that altered expression of WT1 may play a role in prostate cancer development, and that in prostate cancer, WT1 may function other than as a classical tumor suppressor.

*Keyword:* **Truncated WT1 transcript; Prostate cancer**

## Introduction

The Wilms' tumor suppressor gene (WT1) encodes a transcription factor of the zinc finger family (1,2) that plays a major role in both normal genitourinary development (3,4) and the growth of Wilms tumor (5, 6). Until recently, little evidence for involvement of WT1 in prostate cell biology or cancer had been reported, although WT1 overexpression and/or point mutations have been detected in acute leukemias (7), as well as melanoma (8), mesothelioma (9), ovarian cancer (10) and breast cancer (11).

We have identified a novel truncated WT1 transcript (12) in a series of prostate cancer cell lines derived from human benign prostate epithelium immortalized with SV40TAg (P69SV40TAg) (13, 14). This aberrant transcript contains only the 3' portion of the WT1 gene (exons 6-10) and a portion of intron 5 at its 5' end (12). This truncated transcript presumably arises as a result of ectopic promoter activity or splice variation, since no abnormality of the WT1 gene could be demonstrated. In this regard the truncated transcript is distinctly different than that reported by Algar and coworkers, which resulted from a rearrangement of the WT1 gene (15). Interestingly, the tumorigenic sublines expressed greater amounts of the truncated WT1 transcript than the parental immortalized cell line. These results led us to evaluate the expression of WT1 transcripts in human prostate cancer.

In this study, our objectives were to analyze human microdissected benign and malignant prostate glands for expression of (1) the novel WT1 transcript recently described and (2) the wild type WT1 transcript in primary prostate cancer specimens obtained by radical prostatectomy. In addition, we explored whether expression of two known targets for WT1 mediated repression or activation, the epidermal growth factor receptor (EGFR) (16) and the insulin-like growth factor type 1 receptor (IGFR-1) (17, 18), was altered.

## Material and Methods

Prostate Tissue Specimens

Residual prostate tissue was obtained from prostatectomy specimens from consenting patients undergoing treatment by physicians of Virginia Urology center, Richmond, Virginia. All specimens were collected under an IRB-approved protocol. Tissue received in the pathology laboratory after surgery was immediately frozen in liquid nitrogen and stored at -80°C until analysis. Pieces of frozen tissue were sectioned at 5 μM intervals on the cryostat, briefly stained with hematoxylin and eosin, and examined microscopically to identify areas of benign and malignant glands within tissue from each individual for subsequent microdissection. The mean combined Gleason score was 6.3 (±0.85). Patients ranged in age from 51-71yrs (median of 62) and included 8 African Americans and 24 Caucasian Americans.

Laser Capture Microdissection and RNA extraction:

Slides containing sections (5 μM thickness) of each specimen were prepared and maintained at -20°C. The malignant and benign glands were identified and microdissected individually using a Pixcell II Laser Capture Microdissection System (Arcturus Corp., Mountain View, CA) (19). For each sample, sections were captured using a 30 μM diameter laser beam. Typically, 1000-2000 shots were taken per cap. Total RNA was isolated from the tissue by acid guanidinium-thiocyanate-phenol-chloroform extraction protocol (20). Sections of tissue from areas of cancer or BPH were removed by placing the tissue-containing cap on a 0.5 ml eppendorf tube containing 200 μl of denaturing solution (guanidium-isothiocynanate) and inverting the tube gently for 2 minutes. The solution was kept on ice during the procedure. At the end of tissue collection, 200μl of phenol-chloroform were added and the tube was vortexed for 2 minutes,

followed by incubation on ice for 5 minutes and then centrifugation at 4° C for 5 minute at 12,000×g. The aqueous phase was then transferred to a fresh tube and 1/10 volume of 3 M sodium acetate and 200µl of acid phenol chloroform (5:1, pH 4.5) were added. The mixture was incubated on ice for 5 minutes and then centrifuged as above. Finally the aqueous phase was transferred to a fresh tube . Equal volumes of 100% isopropanol and glycogen (at final concentration of 0.1 µg/µl) were added to precipitate the RNA. After overnight precipitation at -20°C, the solution was centrifuged at 12,000×g for 10 minutes at 4° C. The RNA pellet was then washed with 100 µl of 100% cold ethanol and the pellet was recovered by centrifugation at 7,000×g for 5 minutes. The pellet was dried and RNA was dissolved in 15 µl of RNase-DNase free water containing RNase inhibitor (RNAsin) (Promega, Madison, WI) at a concentration of 1 U/µl.

RT-PCR

1.75 µl of total RNA isolated from microdissected tissue was used in a reverse transcriptase master mix consisting of 75 mM KCl, 50 mM Tris (pH 8.3), 3.0 mM $MgCl_2$, 833 µM dNTPs and 2 µM primers and 4.2 U/µL Moloney Murine Leukemia Virus (M-MLV) RT (Gibco/BRL, Rockville, MD). The RT step was performed at 42°C for 1 hour followed by MMLV heat inactivation at 95°C for 5 min. The cDNA was then combined with a PCR master mix with a resulting concentration of 56 mM KCl, 19.6 mM Tris (pH 8.3), 1.6 mM $MgCl_2$, 200 µM dNTPs, 1µM of primers and 2.5U/100µl of AmpliTaq Gold DNA Polymerase (Perkin Elmer, FosterCity, CA). Forty cycles of PCR were performed, using 95°C for 30 seconds for denaturation, 60°C for 30 sec for annealing, and extension at 72°C for 1 min. The PCR products were analyzed by electrophoresis through 1.5% agarose gel at 100 V for 3 hours, then transferred

to Zeta probe membrane (Bio-Rad Laboratory, Hercules, CA), crossed linked by UV light [Stratagene UV cross linker (Stratagene, La Jolla, CA)]. Filters were then hybridized with oligonucleotide probes end labeled with $^{32}P$ γ-dATP (3,000 Ci/mmol) (NEN Life Science Product, Inc., Boston, MA) at 50°C annealing temperature for 6-12 hours. Autoradiographs were performed for 24-72 hours.

Primers and probes, which are used in these experiments, are shown in Table 1. The full length wild type WT1 transcript was reverse transcribed and amplified with primer set 1 and detected with the probe number 2. (Table 1). The short WT1 transcript was reverse transcribed and amplified with primer set 3 and detected with probe number 4. The EGFR transcript was reverse transcribed and amplified with primer set 5 and detected with probe number 6. The IGFR-1 transcript was reverse transcribed and amplified with primer set 7 and detected with probe number 8. As an internal control, the β-actin transcript was reversed transcribed and amplified with primer set 9 and detected with probe number 10

**Table 1-Primers and Probes**

| | Function | Sense Primer or Probe | Antisense | Position (1) | Size -bp |
|---|---|---|---|---|---|
| 1 | RT-PCR exon 1-2 | 5'-TGA GCG CCT TCA CTG TCC AC-3' | 5'-GAA GGT GAC CGT GCT GTA AC-3' | 646-842 | 197 |
| 2 | Probe in exon1 | 5'-CCG GCT GTG CCA GTG AAC-3' | | 695 | |
| 3 | RT-PCR intron 5-exon 6 | 5'-GAA CCC TGC ATC TAA AGT GG-3' | 5'-CGT TGT GTG GTT ATC GCT CT-3' | -159-1214 | 95 |
| 4 | Probe in exon 6 | 5'-CCA CAG CAC AGG GTA CGA-3' | | 1178 | |
| 5 | RT-PCR for EGFR | 5'-GGT GAC TCC TTC ACA CAT AC-3' | 5'-TTG GTC CTG CCG CGT ATG AT-3' | 1318-1478 | 161 |
| 6 | Probe for EGFR | 5'-CCA CAG GAA CTG GAT ATT CT-3' | | 1351 | |
| 7 | RT-PCR for IGFR-1 | 5'-GGG AAT TCC CCG ACC TCG CTG TGG GG-3' | 5'-GGA AGC TTG GAA CAG CAG CAA GTA CTC-5' | 70-310 | 255 |
| 8 | Probe for IGFR-1 | 5'-ATC GAC ATC CGC AAC GAC TAT CAG CAG CTG AA-3' | | 154 | |
| 9 | RT-PCR for actin | 5'-TCT CGC AGC TCA CCA TGC-3' | 5'-CCA CAT AGG AAT CCT TCT GA-3' | | 177 |
| 10 | Probe for actin | 5'-GTC GTC GAC AAC GGC TCC GGC ATG-3' | | | |

*Note* (1) Numbering of the corresponding WT1 cDNA sequence refers the published sequence [Gessler et al., 1992 (21)]; Numbering of the corresponding EGFR cDNA sequence refers the published sequence [Ullrich et al., 1984 (22)]; Numbering of the corresponding IGFR-1 cDNA sequence refers the published sequence [Ullrich et al., 1986, (23)].

**Results**

Either the wild type WT1 transcript or the novel truncated WT1 transcript was found in the microdissected malignant prostate glands of 18/32 cases analyzed by RT-PCR, as summarized in Table 2. The truncated WT1 transcript, consisting of a portion of intron 5 and exons 6-10, was detected in the malignant glands, but not in benign glands, in 5/32 cases (Figure 1). The novel transcript was found in both morphologically benign and malignant glands from a single case (number 99), and seen in the benign glands, but not the corresponding tumor, in only one case (number 94). Expression of the truncated WT1 transcript was significantly associated with malignant morphology ($p<0.03$ Fisher's Exact Test). Full-length WT1 transcripts were restricted to malignant glands in 10/30 cases tested, and seven of these are illustrated in figure 2. However, there were 5 cases in which the wild type WT1 transcript was detected in both malignant and benign glands of the same case. In 4/30 cases, wild type WT1 transcript was seen in the morphologically benign glands, but was absent from the tumor in these cases. Expression of the wild type transcript was significantly associated with malignant morphology ($p<0.05$ Fisher's Exact Test).

There was no correlation between the expression of either wild type or truncated WT1 and the combined Gleason Score or the pre-operative serum PSA level. The mean combined Gleason Score for those with normal WT1 expressed in the malignant glands was 6.0(±0.5); for those without WT1 expression, the mean combined Gleason score was 6.7 (±0.9). Similarly, the mean combined Gleason Score for those tumors expressing the truncated WT1 was 6.5 (±0.7), as compared to a mean score of 6.2 (±0.9) for those not expressing the transcript.

We examined seven cases of prostate cancer that expressed the wild type WT1 transcript and ten cases that did not express WT1 to determine whether the expression of WT1 was associated with differences in expression of EGFR or IGFR-1 transcripts. The malignant glands of 6/7 WT1 (wild type) positive cases tested were also EGFR positive, and all seven produced

IGFR-1 transcripts. (See Figure 3). This is comparable to the results obtained in analyses of the WT1 negative cases: 8/10 were EGFR positive and all ten were IGFR-1 positive.

**Discussion**

Over half of the primary prostate cancers investigated expressed either the wild type or the recently described novel truncated transcript of WT1 (12), as summarized in the Table 2. Expression of either WT1 transcript, the wild type or the truncated transcript, was significantly associated with malignant morphology ($p< 0.05$ and $p<0.03$, respectively). These results suggest that altered expression of WT1 may play a role in prostate cancer development. In addition, identification of the truncated WT1 transcript in primary malignant prostate cancer cells extends our earlier observation of it in two primary leukemias (12), and further demonstrates that the novel transcript is not an artifact of cell culture conditions in the prostate cancer cell lines in which it was first discovered. In addition, these findings indicate that prostate cancers, like some breast (11) and ovarian cancers (10), may be accompanied by overexpression of the WT1 gene. However, expression of WT1 was not confined exclusively to the neoplastic glands. Both the truncated and the wild type WT1 transcripts were detected in a small number of benign glands surrounding neoplasms (Table 2).

The role of either the novel truncated or wild type WT1 gene product in prostate cancer development is not clearly understood. Based on the structure of the truncated form inferred from sequencing data (12), the truncated polypeptide would lack most of its N terminus, where transactivation and self-oligomerization domains reside. However, the truncated form of WT1 described in this report retains the DNA binding domain, and thus could bind to the promoters of WT1 target genes.

Both EGFR (13) and IGFR-1 (24) expression decreases as human prostate cancers progress, and both EGFR (16) and IGFR-1 (17, 18) can be repressed by the WT1 gene product. Decreased expression of WT1 and increased expression of insulin-like growth factor type I (IGF-

I) receptors have been reported in stromal cells from benign hyperplastic prostate tissue compared with stromal cells from normal or malignant prostate (25). Therefore, it is interesting that most malignant glands expressing the wild type WT1 also expressed transcripts for EGFR and IGFR-1, as detected by RT-PCR with appropriate primers. This could indicate that the wild type WT1 protein is not responsible for the reduction in IGFR-1 transcripts (26) or EGFR protein (27) reported by other investigators and ourselves. However, our RT-PCR was not quantitative, and is possible that quantitative differences in the transcription of these growth factor receptors may occur. This is currently under investigation in our laboratories.

At this juncture, we do not know whether there is any prognostic significance attributable to expression of either the full length or truncated WT1 transcript. However, based on the structural information available for the truncated WT1 transcript, we have demonstrated that our assay can detect the novel truncated form against the background of the full-length WT1 transcript. This approach will provide a sensitive method for monitoring WT1 and WT1 variant expression in even small prostate tumor specimens, thereby facilitating the type of outcome analysis necessary to evaluate biological significance in the context of tumor progression.

**Conclusion**

This report demonstrated that the expression of truncated WT1 and wild type WT1 transcript can be detected in a subset of human prostate cancer by using microdissection and RT-PCR on the frozen specimens. A significant association between overexpression of truncated WT1 and malignant morphology was demonstrated. This result suggests that WT1 alteration may contribute to the development or progression of prostate cancer, and that the expression of the truncated WT1 may play a novel role in prostate carcinogenesis. From a technical standpoint, the ability to discriminate between the truncated WT1 transcript and any contaminating genomic DNA provides a strategy suitable for monitoring expression of the truncated transcript in a clinical context.

**Table 2:** Summary of RT-PCR result of wild type and truncated WT1 transcripts expression in human primary prostate cancer

| Clinico-Pathological Data | | | | | RT-PCR of WT1 | | | |
|---|---|---|---|---|---|---|---|---|
| Sample # | Age | Race | Gleason Score | PSA Level | Wild Type | | Truncated | |
| | | | | | B | T | B | T |
| 28 | 60 | CA | 6 | 5.2 | - | - | - | + |
| 35 | 61 | CA | 6 | 1.2 | - | - | - | + |
| 40 | 70 | AA | 5 | 1.6 | ND | ND | - | - |
| 45 | 66 | CA | 7 | 4.73 | ND | ND | - | + |
| 60 | 60 | CA | 6 | 4.16 | + | - | - | - |
| 62 | 58 | CA | 6 | 4.1 | - | + | - | - |
| 19 | 62 | CA | 5 | 4.44 | - | + | - | - |
| 22 | 70 | CA | 8 | 12.5 | - | - | - | - |
| 96 | 57 | AA | 7 | 4.9 | - | - | - | - |
| 24 | 59 | CA | 6 | 7.7 | - | + | - | - |
| 112 | 66 | AA | 7 | 10 | + | + | - | - |
| 123 | 56 | AA | 7 | 19 | - | + | - | - |
| 57 | 66 | CA | 5 | 4.4 | + | + | - | - |
| 63 | 59 | CA | 5 | 4.8 | - | - | - | - |
| 70 | 63 | CA | 6 | 5.8 | + | + | - | - |
| 76 | 63 | CA | 7 | 2.4 | - | - | - | - |
| 130 | 58 | AA | 7 | 12.6 | - | - | - | - |
| 138 | 60 | AA | 5 | 6.2 | - | - | - | - |
| 68 | 69 | CA | 7 | 5.8 | - | - | - | - |
| 85 | 62 | CA | 6 | 10.1 | - | + | - | - |
| 86 | 68 | CA | 5 | 7 | - | + | - | - |
| 89 | 66 | CA | 4 | 4.47 | + | - | - | - |
| 91 | 70 | CA | 8 | 7 | + | - | - | - |
| 141 | 59 | AA | 7 | 5.1 | - | - | - | - |
| 154 | 62 | AA | 9 | 7.09 | - | - | - | - |
| 92 | 66 | CA | 6 | 9.8 | - | + | - | - |
| 94 | 69 | CA | 6 | 9.3 | - | + | + | - |
| 95 | 63 | CA | 8 | 7.34 | - | + | - | + |
| 98 | 60 | CA | 6 | 4.2 | + | + | - | - |
| 99 | 62 | CA | 6 | 1.2 | + | + | + | + |
| 100 | 71 | CA | 6 | 13.27 | - | + | - | + |
| 75 | 51 | CA | 6 | 14.3 | + | - | - | - |

*B = benign glandular epithelium, T= malignant glandular epithelium, ND= study was not successful due to diminished integrity of RNA, CA= Caucasian American, AA = African American

**Figure legends**

**Figure 1**: RT-PCR product using primers in intron 5 and exon 6 for (Table 1) detecting truncated WT1 transcript in primary prostate cancer. Total RNA was obtained from primary prostate cancer specimens. Twenty five microliters of reaction products were examined on 1.5% agarose gel, transferred to nylon membrane and probed with an oligonucleotide in exon 6 (Table 1; # 4). The 95 bp fragment represents amplified products from truncated WT1 transcript whereas the 196 bp product represents amplified products from contaminating genomic DNA. Sources of RNA are indicated as sample number in the top row of the table. B=> benign, T=> malignant epithelium. RT-PCR for actin transcripts was performed as a control in all cases.

**Figure 2**: RT-PCR product using primers in exon 1 and exon 2 (Table 1) for detecting full length WT1 transcript in primary prostate cancer. Total RNA was obtained from primary prostate cancer specimens. Twenty five microliters of reaction products were examined on 1.5% agarose gel, transferred to nylon membrane and probed with an oligonucleotide in exon 1 (Table 1; # 2). The 197 bp fragment represents amplified products from full length WT1 transcript. Source of RNA are indicated as sample number in the top row of the table. B=> benign, T=> malignant epithelium. RT-PCR for actin transcripts was performed as a control in all cases.

**Figure 3**: RT-PCR products using primers for detecting growth factor receptor (EGFR and IGFR-1) transcripts in primary prostate cancer. Total RNA was obtained from primary prostate cancer specimens. Twenty five microliters of reaction products were examined on 1.5% agarose gel, transferred to nylon membrane and probed with an oligonucleotide for EGFR (Table 1; # 6) and IGFR-1 transcripts(Table 1; # 8). The 161 and 255 bp fragments represent amplified products from EGFR and EGFR-1 transcripts respectively. Source of RNA are indicated as sample number in the top row of the table. B=> benign, T=> malignant epithelium. RT-PCR for actin transcripts was performed as a control in all cases.

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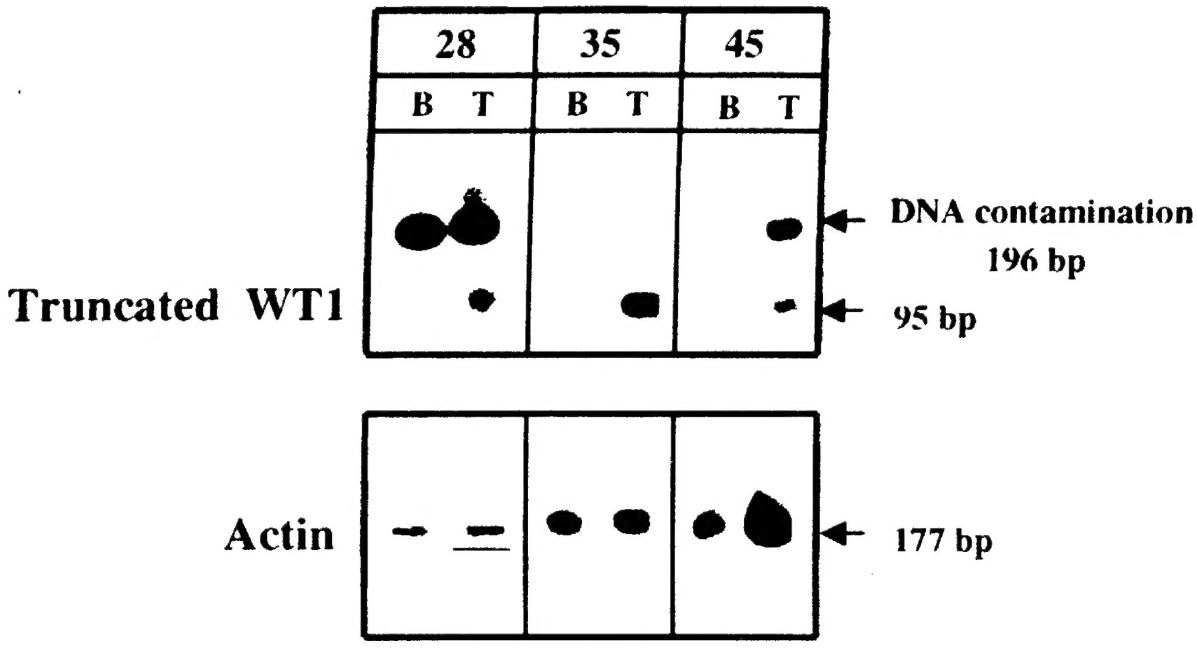
FIG 1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35
45
B T
B T
B T
Truncated WT1
DNA contamination
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95 bp
Actin
177 bp

Fig 2

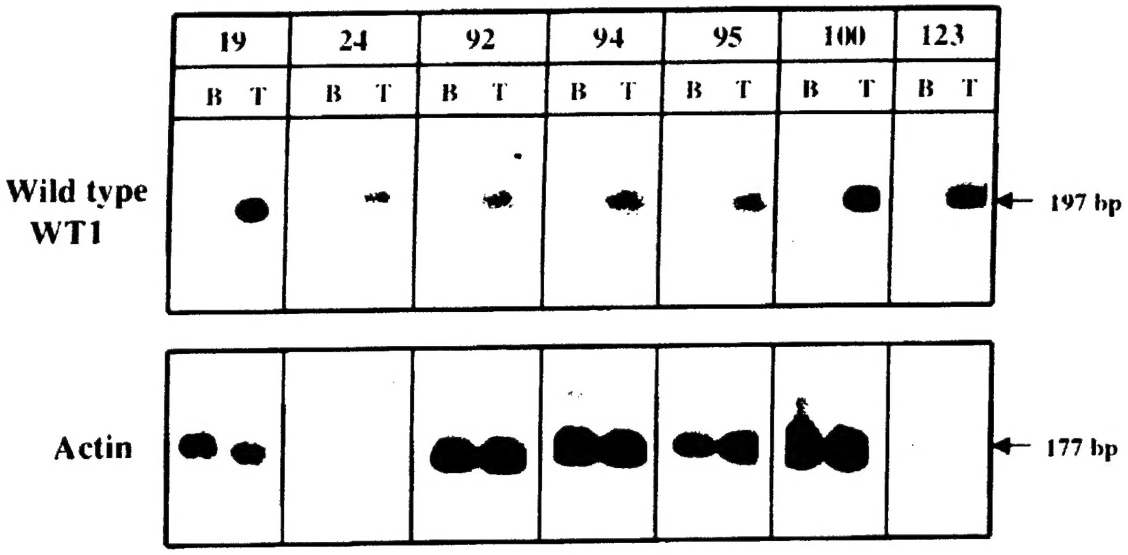
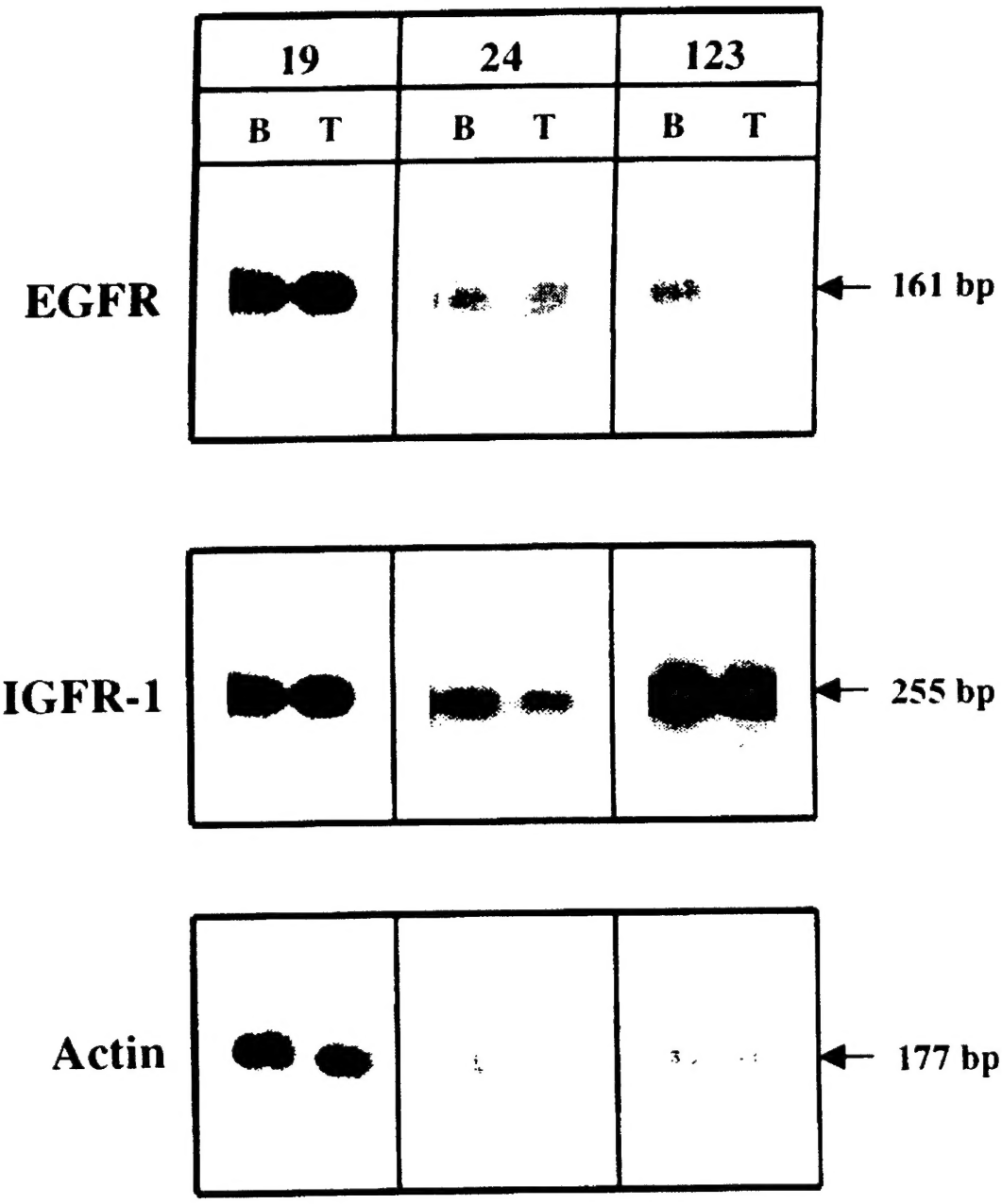


Fig 3



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Catherine I Dumur
Pathology Department
Virginia Commonwealth U
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A REAL-TIME RT-PCR ASSAY FOR QUANTITATION OF DIFFERENT WT1 TRANSCRIPTS IN CANCER.


Dumur CI, Deschsukhum C, Garrett CT, Wilkinson DS, Ware JL and Ferreira-Gonzalez A. Department of Pathology VCU, Richmond, VA 23298



The Wilm's tumor suppressor gene (WT1) modulates several growth factors and their receptors that have been implicated in prostate cancer development. We have recently demonstrated differential expression of wild type (WT) and a truncated (TR) transcript lacking the first 5 exons, among human prostate cancer cell lines. Our objective was to develop and validate a real-time RT-PCR assay for the quantitation of these two WT1 transcripts. We developed two WT1 transcript-specific in vitro-generated RNA as calibrators. Serial dilution of these calibrators demonstrated a 5 log linear dynamic range ($5x10^1$ to $5.10^6$ copies/reaction, $R^2$= 0.9964 for WT and $R^2$= 0.9975 for TR). Dilution of the calibrators in total RNA from $1x10^3$ non WT1-expressing cells, showed decreased sensitivity without affecting the linear dynamic range. Precision studies for values within the linear dynamic range showed a coefficient of variation of less than 6% for both transcripts. Real-time quantitative PCR provided a sensitive and reliable method for quantitating both of these WT1 mRNA species in cell lines and prostate cancer tissue samples. (DAMD 17-98-1-8540 and RO1 DK 52683)


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Association for Molecular Pathology
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